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Detection of Cytokeratin-Positive Cells in Bone Marrow in Breast Cancer and Colorectal Carcinoma in Comparison with Other Factors of Prognosis

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ABSTRACT

A prospective study is presented in which 293 patients suffering from breast cancer and colorectal carcinoma were analyzed for prognostic relevance of detected isolated disseminated tumor cells in bone marrow (IDTBM). The patients underwent surgery in the period from 1995 to 1997 and remained under observation until 1999. The monoclonal antibody A 45-B/B3 was used in the standard immuno-cytochemical method for detecting IDTBM, which represented an independent prognostic factor for survival time in patients with breast cancer or colorectal cancer. In breast cancer, when IDTBM were detected, the survival period was reduced by at least half. When disseminated tumor cells containing the A45-B/B3 antibody were detected in bone marrow, the risk of an earlier relapse of the tumor increased at least fourfold. In colorectal cancer, detection of IDTBM reduced survival time by a factor of 1.2–4.3. The risk of earlier relapse increased when disseminated tumor cells containing the A45-B/B3 antibody were detected in bone marrow by 2.8–8.1. Therefore, the use of IDTBM as an independent prognostic factor would provide an important method for determining the pathological stage of various cancers. Standardization of this technique into a generally accepted method would be especially desirable in treatment of patients with breast or colorectal cancer.

INTRODUCTION

DESPITE USE OF RADICAL AGGRESSIVE THERAPEUTIC METHODS, distant metastases develop in approximately 50% of carcinoma patients within 5 years, in part because conventional methods of diagnosis do not give any indication of metastases at the time of surgery. While, the R0 method of resection of solid tumors is effective, local relapses are the main concern of the surgeon and distant metastases have been traced back to systemic dissemination of tumor cells at the time of surgery (1).

Simple detection of tumor cells in the region of venous drainage from tumors into the systemic circulation were known in the 1950s; in the 1960s and 1970s, large-scale investigations were undertaken to detect primary tumor cells that had metastased into bone marrow by using conventional staining (2). The immunocytochemical analysis of bone marrow aspirates was enabled by the development of immunohistochemistry since 1980. Over the following 10 years, several groups developed, validated the clinical relevance, and distinctly increased the sensitivity of tumor cell detection (3–6). This is currently considered the standard method of detecting occult early dissemination of solid tumor cells (7,8). "Minimal residual disease" and "isolated disseminated tumor cells in bone marrow" (IDTBM) have become generally estab-

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TABLE 1. DESCRIPTION OF PATIENTS AND STAGE OF DISEASE

	Breast cancer	*Colorectal carcinoma*
Number of patients	126	167
Average age	65	66
Lymph node metastases	47 (37.3%)	83 (49.7%)
Distant metastases	7 (5.5%)	43 (25.7%)
Local R0 resection	120 (95.2%)	120 (71.9%)
Local R1/2 resection	6 (4.8%)	47 (28.1%)
Mean time of postoperative observation (months)	42 (12–72)	37 (12–62)

lished terms leading to the International Union against Cancer (UICC)'s introduction of an M1(i) stage (1). These investigations showed that the presence of disseminated tumor cells in bone marrow indicated a worse prognosis for the patient concerned. Findings regarding the prognostic importance of detecting IDT in lymph nodes are still contradictory. The significance of IDT in fluid from peritoneal lavage and venous blood has not been studied extensively enough to indicate its utility as a prognostic indicator (9–12).

Our 6-year study attempted to evaluate the significance of the IDTBM as a means of prognosis of patients with breast cancer, colorectal carcinoma and gastric carcinoma, as compared with established means of prognosis, and to evaluate the effectiveness of the technique.

PATIENTS AND METHODS

Patients and sampling method

We present a prospective study involving 293 patients with tumors in various stages of advancement, who underwent tumor resection between 1994 and 1997 and were observed until 1999. The study group was comprised of 126 breast cancer patients and 167 patients with colorectal carcinoma (Table 1). Immediately after surgery, while the patient was still anesthetized, the iliac crest on both sides of the pelvis was punctured by the customary method, and 5 ml of bone marrow aspirate was obtained from each side. The aspirate was used to detect any residual disease after the tumor resection including tumor cells that were shed during surgery. The R classification of patients is related exclusively to the local tumor situation.

Immunocytochemistry

The marrow samples were analyzed separately in left versus right iliac crest aspirates. In four cyto-centrifugation steps we separated the cells from the bone marrow aspirates using Ficoll–Paqued density centrifugation and prepared slides by cytospin. We adjusted the cell concentration to obtain 100,000 cells per microscope slide and analyzed 1 million cells per patient (13,14). The immuno-cytological identification was achieved through the Epithelial Cell Detection Kit (EPIMET®), Mikromet GmbH/Baxter Deutschland GmbH), which contains a monoclonal pancytokeratin antibody against A45-B/B3. The identification of epithelial cells is based on the reactivity of the murine monoclonal antibody A45-B/B3 with the epithelial cell's cytoskeleton. The EPIMET® method involves adhesion of cells to glass slides, permeabilization and fixation of cells, binding of cytoskeletal cytokeratins with a conjugate of a specific Fab fragment and alkaline phosphatase, and formation of an insoluble red reaction product at the binding site to produce a brilliant red color in positive cells (Fig. 1).

For quality control we used a negative control without antibodies, a positive control for staining, and a positive control of a histopathologically positive breast carcinoma section. Fifty-one patients without detectable carcinoma were examined for IDTBM by using the same method. None of the patients from the control group tested positive for IDTBM with the A45-B/B3 antibody.

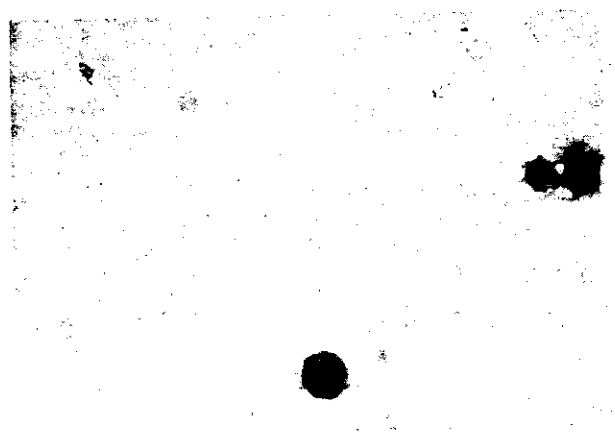

FIG. 1. Cytokeratin-positive cell with the A45-B/B3 antibody.

TABLE 2. UNIVARIATE ANALYSIS OF SURVIVAL TIME ACCORDING TO KAPLAN–MEIER BREAST CANCER

Factor of prognosis	*Influence on survival time*	*Influence on time until progression*
A45-B/B3 positiveness	0.004	<0.001
pT state	<0.001	<0.001
pN state	0.0032	<0.001
M state	<0.001	<0.001
R classification	<0.001	<0.001
Age	0.0663	0.1561

Statistical analysis

Cell preparations were examined by transmission light microscopy by two independent researchers. The Wilcoxon test, Chi-square test, Fisher's Exact, Kaplan–Meier–Schätzer, and log rank test were used to statistically evaluate results. The Cox regression was used for analysis of tumor size, lymph node involvement, and distant metastasis. The nature of the study under consideration is subjective, but the effects and results are significant. One of the problems with statistical analysis of this study lies in the multitude of tests performed on the same set of data. The problem of multiple testing can be circumvented by adjusting the level of significance for each test so that each test is significant only when the p-value is <0.05 times the number of tests. Therefore, for 10 tests, each individual test is significant if $p < 0.005$. The level of significance for the study will thus be kept at 5%.

RESULTS

Comparison between sides of the iliac crest in the same patient showed not noticeable difference in cytokeratin reactivity. When positive results were seen, as few as 1 cell, or as many as 500 IDTBM (in one patient) were detected in 10^6 mononuclear cells, After the univariate analysis, the following prognostic factors were included in the Cox regression: the epimet positiveness, tumor size (pT1-2/3–4), involvement of lymph nodes (pN0/pN$^+$) and distant metastases, whether primary illness or relapse (R classification R0/R1-2), and age (dichotomized by the group median).

Breast cancer

In tests with the A45-B/B3 antibody, 15.9% (20/126) of the patients were cytokeratin positive. Correlation was indicated between positive reactions to A45-B/B3 antibody, lymph gland metastases ($p < 0.001$, and diagnosed distant metastases (Fisher's exact test P: 0.015). A correlation between the size of tumor and grading was not detectable.

In the univariate survival time analysis (Table 2) a correlation between cytokeratin reacitivity and survival time ($p = 0.004$) was indicated. Conventional prognosis methods showed significant correlation between tumor size and survival time, and between tumor size and renewed progression of the tumor ($p < 0.001$). Correlation was shown also between lymph node involvement (pN stage) survival time ($p = 0.0032$), and the time until progression of the tumor ($p < 0.001$). Correlation between M stage and survival time and the time until tumor progression also produced a significance level of $p < 0.001$. The importance of surgery to prevent local relapse could be established for survival time in cases of carcinoma of the breast ($p = 0.3037$), however, time until progression of the tumor indicated more importance ($p = 0.0013$). A palliative or curative approach of therapy, although determined by the stage of the tumor, had a significant influence on the survival time of patients ($p = 0.0003$) and, more obviously, on the time before progression of the carcinoma was observed ($p < 0.001$). In younger patients (dichotomized by the group median), the time of survival was shorter ($p = 0.0663$).

Multivariate analysis (Table 4) showed that detection of isolated disseminated tumor cells ($p = 0.0049$) represented an independent prognostic factor for survival time. None of the other factors that we examined demonstrated this prognostic relevance. The risk of earlier death increased at least twofold (RR value: 11.5, confidence interval: 2.1 to 62.9), with detection of cytokeratin positive cells by reaction with A45-B/B3 antibody in bone marrow as compared with cytokeratin-negative patients. Demonstration of progression of the tumor through local relapse ($p < 0.001$) and the detection of isolated disseminated tumor cells ($p < 0.001$) represented independent prognostic factors. With breast cancer, the risk of an earlier (renewed) progression of the tumor increased by 10.3 in patients who had suffered a local relapse (RR value), at a confidence interval of 3.1 to 34.1, as compared with patients with a first diagnosis of breast cancer; detection of disseminated tumor cells in bone mar-

TABLE 3. UNIVARIATE ANALYSIS OF SURVIVAL TIME ACCORDING TO KAPLAN–MEIER COLORECTAL

Factor of prognosis	*Influence on survival time*	*Influence on time until progression*
A45-B/B3 positiveness	0.0058	<0.001
pT state	<0.001	<0.001
pN state	<0.001	<0.001
M state	<0.001	<0.001
R classification	<0.001	<0.001
Age	0.0367	0.116

row by the A45-B/B3 antibody indicated an increase in risk by 13.5 (RR value) at a confidence interval of 4.2 to 43.2 when compared to cytokeratin-negative patients. Patients with both local relapse and disseminated tumor cells have, on an average, approximately a 100 times greater risk of early progression of tumor growth.

Colorectal carcinoma

With the A45-B/B3 antibody, 24% (40/167) of colorectal carcinoma patients were cytokeratin positive. No correlation was detectable between a positive test for cytokeratin and tumor size, lymph node involvement, or formation of distant metastases. In univariate analysis (Table 3), a correlation was seen between positive cytokeratin and survival time ($p = 0.0058$) and, also between positive cytokeratin and, the time before progression of the tumor (p-value < 0.001). Considering the conventional prognosis factors, there was a significant correlation between size of tumor and survival time and between tumor size and time until progression (Table 2) (p-value < 0.001). Correlation occurred between lymph node infestation (pN stage) and survival time (p-value < 0.001) and between lymph node involvement and the time until progression of the tumor (p-value < 0.001). The same level of significance, <0.001 was seen in the correlation between M stage and survival time and between M stage and the time until the tumor progressed. The significance of surgery for local relapses influenced survival time (p-value < 0.001) and time until the tumor progressed (p-value < 0.001). A palliative or curative approach of therapy, although determined by the stage of the tumor, had significant influence on the survival time of patients (p-value < 0.001) and on the time until the progression of the carcinoma (p-value < 0.001).

In the multivariate analysis (Table 5), detection of isolated disseminated tumor cells ($p = 0.0090$), detection of lymph node metastases ($p = 0.0088$) and detection of distant metastases ($p < 0.001$) on survival time were independent prognostic factors. The risk of earlier death increased with the detection of cytokeratin positive cells in the bone marrow as detected by the A45-B/B3 antibody by 2.3 (RR value) at a confidence interval of 1.2 to 4.3, as compared with cytokeratin-negative patients. In combination with the detection of lymph node metastases at the time of surgery, cytokeratin-positive cells in the bone marrow increased the risk of early death by 3.2 times (RR value) at a confidence interval of 1.3 to 7.6, as compared with nodal negative patients. The presence of cytokeratin positive bone marrow cells in combination with the detection of distant metastases at the time of surgery increased the risk of early death by 5.8 (RR value) at a confidence interval of 2.8 to 11.9, as compared with metastasis-free patients. The detection of isolated disseminated tumor cells (p-value < 0.001), the detection of lymph node metastases ($p = 0.0009$) and the detection of distant metastases (p-value < 0.001) were independent prognostic factors also for the time until progression of the tumor. Using the A45-B/B3 antibody to detect cytokeratin positive cells, the risk of an earlier relapse of the tumor increased with the detection of disseminated tumor cells in the bone marrow by 4.7 times (RR value) at a confidence interval of 2.8 to 8.1, as compared with cytokeratin-negative patients; with the detection of lymph node metastases

TABLE 4. MULTIVARIATE ANALYSIS—BREAST CANCER

	Survival time p-value confidence interval (95%)		*Time until progression of tumor p-value confidenceinterval (95%)*	
A45-B/B3 positiveness	0.0049	2.1–62.9	<0.001	4.2–43.2
Local replase	<0.001	3.1–34.1	n.a.	n.a.

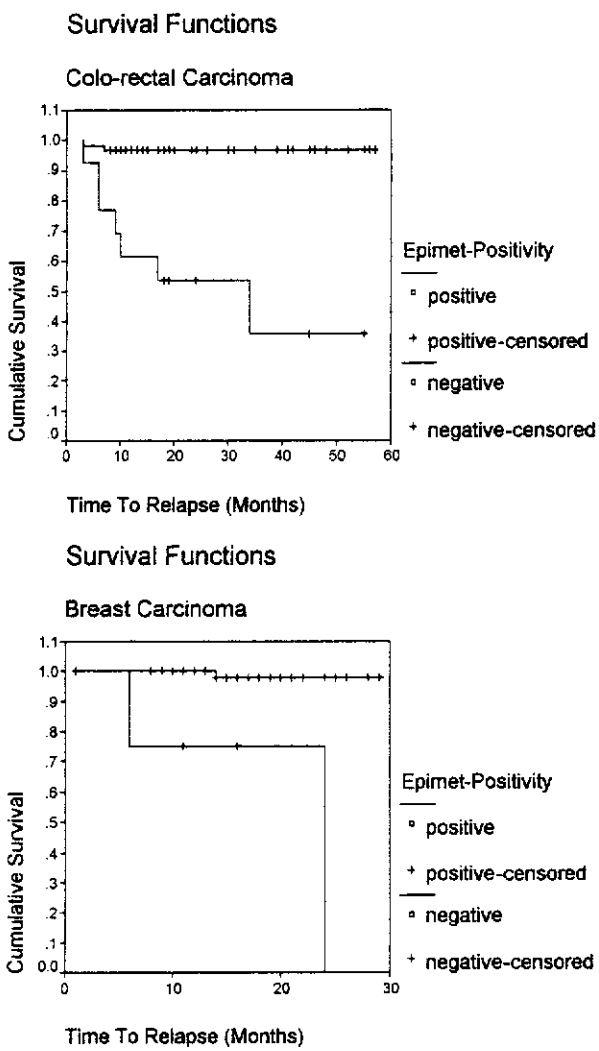

FIG. 2. Kaplan–Meier curves: breast and colorectal carcinoma (time to relapse).

at the time of operation the risk was increased by 3.0 (RR value) at a confidence interval of 1.6 to 5.9, as compared with nodal-negative patients. With the formation of distant metastases at the time of surgery, the risk of early relapse increased by 4.4 (RR value) at a confidence interval of 2.4 to 7.8, as compared with metastasis-free patients.

DISCUSSION

Along with new methods for detecting "minimal residual disease," such as RT-PCR, the immuno-cytochemical detection of isolated tumor cells in bone marrow (IDTBM) by using monoclonal antibodies is currently considered the standard method (1,7). With the A45-B/B3 antibody, 16–24% of the patients in our study were cytokeratin positive. The detection rates mentioned in specialized publications are, on an average, 4–45% (1,7). In a meta-analysis of 20 studies of all types of carcinoma, such cells were detected in an average of 35% of patients (15). In our opinion, the differences in detection rates can be attributed to methodological aspects. Moreover, the studies cannot be directly compared because, on the one hand, polyclonal (EMA) antibodies (16), antimuzin antibodies (2E11) and other antibodies have been used separately from the CK 2 antibody (7). A generally accepted standardization of these methods is urgently required to facilitate a comparison between various studies in the future.

With breast cancer, the simple statistics of median comparison and correlation analysis indicated a correlation between A45-B/B3-detected cytokeratin-positive cells and the presence of metastases in lymph nodes and distant locations. With colorectal carcinoma, there were indications of a correlation between A45-B/B3-detected cytokeratin-positive cells and lymph node metastases. Taken together, a partial correlation was seen between cells that reacted to the A45-B/B3 antibody to show positive cytokeratin and conventional prognosis factors. Many other authors have confirmed a correlation between positive tumor cells in bone marrow and the size of tumor, the state of lymph node metastases and distant metastases and other prognostic factors (17,18). Still other authors observed a tendency for such correlation, but were unable to provide significant evidence (8).

The next step for assessing the prognostic relevance of IDTBM are univariate analyses with respect to total survival time and the time until progression of the tumor. In our study, a similar correlation was found between cytokeratin positiveness and the conventional prognosis factors (size of tumor, status of lymph node, status of distant metastasis) of the TNM systems, irrespective of the

TABLE 5. MULTIVARIATE ANALYSIS—COLORECTAL CARCINOMA

	Survival time p-*value*	*confidence interval (95%)*	*Time until progression of tumor* p-*value*	*confidence interval (95%)*
A45-B/B3 positiveness	0.0090	1.2–4.3	<0.001	2.8–8.1
Lymph node metastases (pN0/pNpositive)	0.0088	1.3–7.6	<0.001	1.6–5.9
Distant metastases	<0.001	2.8–11.9	<0.001	2.4–7.8

kind of carcinoma (Tables 2 and 3). In earlier studies, the prognostic IDTBM value was often not tested. These results also coincide with the prognostic IDTBM relevance established by other studies on a univariate basis (11,19, 20). A few studies could not establish any prognostic relevance of the IDTBM with respect to survival time or the time until progression of the tumor (9,17,18,21–23).

To demonstrate the independence of prognostic factors, univariate analysis determines the significance of each individual factor. On the basis of multivariate analyses, several other authors showed the independent prognostic relevance of IDTBM for survival time and for the period of time until relapse (19,24). The majority of studies where a prognostic relevance for IDTBM was found by a multivariate approach, were concerned with breast cancer; few were concerned with colorectal carcinoma (15). With breast and colorectal carcinoma (Tables 4 and 5), a positive reaction to A45-B/B3 antibody and local relapse of the disease early in the observation period represented independent prognostic factors. The risk of an earlier death was at least doubled with the detection of IDTBM. Detection of IDTBM and, at the same time, occurrence of a local relapse, increased the risk of the progress of a tumor by approximately 100 times in breast carcinoma. Interestingly, all other conventional prognosis factors (TNM system) failed to produce as high a level of prognostic relevance compared with the IDTBM. In the meta-analysis mentioned above, in 2494 randomized breast cancer patients (15) the relative risk of a shortened illness-free interval with the positive detection of IDTBM was 1.27 to 1.42. In the study under consideration this risk was clearly higher, 4.2 to 43.2. In the meta-analysis, only five of the studies using a univariate approach and only two of the studies using a multivariate approach showed that IDTBM represented an independent prognostic factor for total survival time. In a study involving 280 breast cancer patients performed with the antibodies 2E11 and tag 12 rather than A-45-B/B3, the authors (24) showed by a multivariate approach that IDTBM increase the risk of tumor progression by 1.45 to 5.86 and the risk of earlier tumor-related death by 1.58 to 11.2. The prognostic value of this study should be carefully interpreted using the large confidence intervals to indicate expected patient outcomes.

In one of the studies of colorectal carcinoma (5), cytokeratin positiveness (tested with CK18) increased the risk of an early death from the carcinoma by 1.45 to 6.12. In the study under consideration, the risk increased by 1.2 to 4.3 when cells positive for cytokeratin were found (A45-B/B3 antibody).

The involvement of lymph nodes was another independent factor that influenced survival time. The presence of cytokeratin positive nodal cells resulted in an increase of the risk for early patient death by 1.13 to 5.63. In the study reported here, the risk increased by 1.3 to 7.6 for the same factors. For our study, the size of the tumor (pT3-4/1-2, an increase of risk by 1.07–7.07) and the stage of tumor (3–4/1–2, an increase by 1.25–5.09) were additional independent prognosis factors, and distant metastases increased the risk by 2.8 to 11.9.

CONCLUSION

A generally accepted standardization of this method of using IDTBM for prognosis in carcinoma patients is a matter of urgent necessity. This independent prognostic factor is demonstrated here to be an important method to ascertain the pathological stages of tumors. As experience is accumulated from tests for IDTBM, informed decisions can be made whether patients with breast cancer or colorectal carcinoma, who would not normally receive adjuvant therapy because of the stage of their tumor, ought to be advised to undergo this adjuvant therapy solely based on the detection of isolated disseminated tumor cells in their bone marrow. New options for treatment with immunotherapy, which have traditionally been used after metastasis of the carcinoma, are also available for these adjuvant situations. IDTBM would provide another indication for such therapeutic options that could then undergo randomized studies to find the most effective therapy in these cases.

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