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Association of the Presence of Bone Marrow Micrometastases with the Sentinel Lymph Node Status in 410 Early Stage Breast Cancer Patients: Results of the Swiss Multicenter Study

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Background: The sentinel lymph node (SLN) status has proven to accurately reflect the remaining axillary lymph nodes and represents the most important prognostic factor. It is unknown whether an association exists between the SLN status and the presence of bone marrow (BM) micrometastases. The objective of the present investigation was to evaluate whether or not such an association exists.

Methods: In the present investigation 410 patients with early stage breast cancer (pT1 and pT2 ≤3cm, cN0) were prospectively enrolled between 1/2000 and 12/2003. All patients underwent SLN biopsy and bone marrow aspiration. The histological examination of the SLN consisted of step sectioning, H&E, and immunohistochemistry (Lu-5, CK 22) staining. Cancer cells in the BM were stained with monoclonal antibodies A45-B/B3 against cytokeratin and counted by an automated computerized digital microscope.

Results: BM micrometastases were detected in 28.8% (118/410) of all patients. The SLN contained metastases in 32.4% (133/410). Overall 51.2% of the patients (210/410) were SLN negative/BM negative and 12.4% (51/410) SLN positive/BM positive. Of all patients, 16.4% (67/410) were SLN negative/BM positive and 20.0% (82/410) SLN positive/BM negative.

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There was a statistically significant association between the SLN and BM status, both in unadjusted (Fisher's exact test: $P = .004$) and multiple logistic regression analysis ($P = .007$).

Conclusions: In the present investigation a significant association was found between a positive SLN status and the presence of BM micrometastases. Nonetheless, the percentage of nonconcordance (SLN negative/BM positive and SLN positive/BM negative) was considerable. The prognostic impact of BM micrometastases in our patient sample remains to be evaluated.

Key Words: Breast cancer—Sentinel lymph node—Bone marrow micrometastases—Correlation—Multicenter trial.

Disseminated tumor cells in bone marrow aspirates from patients with breast cancer were first detected when applying immunocytochemistry to the specimens.[1] The evidence of a hematogenous spread of tumor cells was interpreted as evidence for a metastatic process. Different studies showed a worse disease-free and overall survival for the subgroups with bone marrow (BM) micrometastases due to a higher risk of developing distant metastases.[2–6] In some investigations, BM micrometastases were described as having the same or even a higher prognostic level compared to axillary lymph node status[2,7,8] and were considered to be a prognostic factor such as tumor size, grading, and hormone receptor status. Therefore, BM micrometastases were thought to be the reason for recurrence and death in node negative breast cancer patients.[9,10] In most of the above mentioned studies the patients underwent a formal axillary lymph node dissection of level I and II with a standard histopathologic examination, i.e. bisection of each lymph node with hematoxylin and eosin (H&E) staining. Nowadays the percentage of node positive patients is constantly decreasing as breast cancer is increasingly detected at an earlier stage. The axillary lymph node status still represents one of the most important prognostic factors in breast cancer patients and determines among others subsequent adjuvant treatment. The sentinel lymph node (SLN) technique proved to accurately reflect the status of the remaining axillary lymph nodes in patients with early stage breast cancer with a very low false negative rate.[11–13] H&E serial sectioning and additional immunohistochemistry of the SLN allowed detecting isolated tumor cells, micrometastases, or even hidden macrometastases.[14] The latter two events led to an upstaging in 10–25% of primarily node negative patients, therefore identifying more truly node negative patients. The objective of the present prospective Swiss Multicenter Study—the first in the literature so far—was to examine the association of the SLN status and the presence of BM micrometastases.

PATIENTS AND METHODS

Between January 2000 and December 2003, a total of 698 patients with early stage breast cancer were prospectively enrolled in the present trial (official name: "Swiss Multicenter Sentinel Lymph Node Study in Breast Cancer Patients"). Inclusion criteria for the present study were: (1) presence of palpable breast cancer, (2) tumor size histologically equal to or less than 3 cm in diameter, (3) absence of clinically palpable axillary lymph nodes, (4) no prior history of breast cancer or other malignancies, (5) no neoadjuvant therapy, and (6) no pregnancy. Thirty-nine patients did not meet the inclusion criteria and were therefore excluded (Fig. 1).

A total of 410 patients agreed to simultaneously undergo a BM aspiration from both iliac crests as the first step of the operation. Written informed consent was obtained from all patients. The other 249 patients refused to give their informed consent for a BM aspiration (Fig. 1). There were no significant differences in the baseline characteristics between the two groups with and without BM aspiration (all P-values $> .05$, data not shown).

The study was approved by all Local Ethical Committees. A total of 13 centers participated in this study, amongst them the Swiss University Hospitals in Basel, Berne, and Zurich, as well as different nonacademic institutions in Switzerland.

Operative Technique on the Primary Tumor

All centers performed breast conserving therapy whenever possible. The breast conserving rate was 90.5% (371/410). The tumorectomy with free surgical margins was the standard technique to remove the primary tumor, usually achieved as a segmentectomy. The extent of the resection was related to the primary tumor size.

Lymphatic Mapping and Operative Technique

SLN mapping was performed by using a combination of a radiolabeled colloid and a vital blue dye.

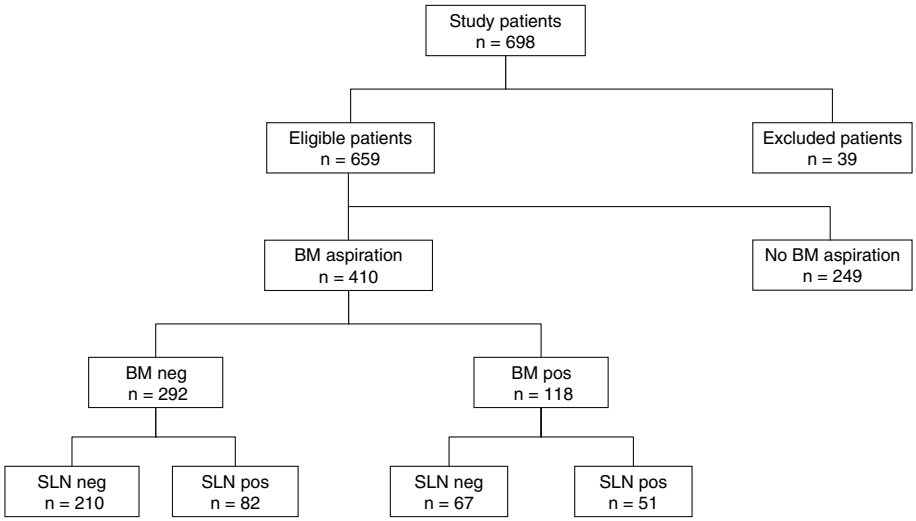

FIG. 1. Study flow chart

^{99m}Tc-labeled nanocolloid (Nanocoll®, Nycomed AG, Wädenswil, Switzerland) at a dose of 70 MBq was injected, peritumorally at four places, whereas at the injection site closest to the axilla half of the dose was injected peritumorally and subdermally. Lymphoscintigraphy was performed preoperatively to identify lymphatic flow to axillary and/or parasternal lymph nodes. Hot spots were marked on the skin. The SLN were intraoperatively identified first by the use of a handheld gamma probe (Navigator®, USSC, RMD Waterton, MA, USA, C-Trak®, Care Wise Medical Products Corp., Morgan Hill, CA, USA, or Neoprobe®, Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, OH, USA). Up to 5 ml of isosulfanblue (Lymphazurin®, Ben Venue Labs Inc., Bedford, OH, USA and Hospital Pharmacy, University Hospital Zurich, Switzerland) or 2–4 ml of patent blue V (Guerbet Group, Roissy, France) were injected 5–10 min prior to incision using the same technique as previously described for the radioactive tracer. Hot and/or blue lymph nodes were excised and labeled as SLN. Dissection was continued until all hot and blue nodes had been removed and the background count of the axilla was less than 10% of the hottest lymph node ex vivo.

Prior to participation to this study, the SLN procedure was validated at all institutions based on at least 20 breast cancer patients in whom both SLN and completion level I and II ALND were performed. A SLN identification rate as well as a sensitivity of at least 95% were sine qua non requirements for participating to this trial. The results of the validation period (20 patients) were critically reviewed by one of the principal investigators (ORK, GB, and MZ). Only after adequate performance was proven, the hospitals were allowed to enroll patients to the present study.

Pathologic Examination of Lymph Nodes

Frozen sections were routinely performed intraoperatively. Lymph nodes larger than 5 mm in diameter were bisected, whereas lymph nodes less than or equal to 5 mm in diameter were not bisected but completely submitted for frozen section analysis. The SLN were intraoperatively examined at three levels with hematoxylin & eosin (H&E) stained sections at a cutting interval of 150 μm. The remaining tissue of the SLN was formalin-fixed and embedded in paraffin for histologic analysis. The residual tissue was then examined using step sectioning at a cutting interval of 250 μm. Step sections were stained with H&E. If no carcinoma cells were detected, immunohistochemistry with cytokeratin antibody Lu-5 or CK 22 using a standard immunoperoxidase method (ABC Elite kit) was performed. Lu-5 (Bio Medicals, Augst, Switzerland) is a pancytokeratin monoclonal antibody that recognizes types I and II cytokeratin subfamilies of all epithelial and mesothelial cells.

Micrometastases are defined based on a size exceeding 0.2 mm and less than or equal to 2 mm in diameter according to the AJCC classification.[15] Therefore, isolated tumor cells (ITC) or tumor cell clusters measuring less than or equal to 0.2 mm in diameter did not meet the definition of micrometastases. Patients with isolated tumor cells (≤0.2 mm) were considered node negative in the present investigation and were analyzed in the node-negative group. Due to the lack of evidence in the literature,

the decision to perform a completion ALND in patients with SLN micrometastases or isolated tumor cells was left to each hospital's directives. No completion ALND was performed in women with tumor-free SLN.

Pathologic Examination of Bone Marrow Aspirates

Bone marrow specimens were processed using Leucosept tubes (Greiner, No. 227290, Solingen, Germany) according to manufacturer's guidelines. Briefly, Leucosept 50 ml tubes were filled with 15 ml histopaque and centrifuged for 1 min at 1,000*g*. A 10–30 ml of the BM specimen were poured on the filter disk and centrifuged 15 min at room temperature. After centrifugation the fat and plasma layers were removed and discarded. Remaining mononuclear cells on the filter plate were diluted with buffer in a ratio of 1:5. This procedure was followed by a centrifugation for 10 min with 800*g*. The supernatant was discarded. Erythrocytes were lysed with lysis buffer. After cell counting, the cells were resuspended and centrifuged. Final concentration was set to 2 million BM cells per milliliter. This step was followed by an incubation with the EPIMET Epithelial Cell Detection Kit (Micromet, Martinsried, Germany) for the identification of epithelial cells. This kit contained monoclonal antibodies A45-B/B3 against cytokeratin 8, 18, and 19 (binding of a conjugate of specific Fab fragments and alkaline phosphatase to cytoskeletal cytokeratins). Fuchsin was given to form a red insoluble reaction product. Subsequently, the preparations were examined using the ACIS® Chroma Vision Automated Cellular Imaging System (San Juan Capistrano, CA, USA) equipment. One or more detected tumor cells were considered as BM micrometastases positive. All preparations were reviewed by one pathologist (GS). The specimen were spiked with the T47D/MCF-7 cancer cell line, which served as a positive staining control. Specimen with a conjugate of Fab fragment of a monoclonal antibody with irrelevant specificity were used as a negative control to exclude unspecific binding of the ab-enzyme complex to the test sample. The positive and negative controls were used with each batch run and were also systematically reviewed by the pathologist to determine the result of a patient's specimen. Therefore, each cytokeratin positive cell being of epithelial origin was exactly classified as a tumor cell or a benign epithelial cell. As the presence of tumor cells in BM aspirates represents a sign of tumor spread, every single tumor cell detected in the BM was regarded as pathologic.

Statistical Analyses

Fisher's exact test was used for unadjusted comparisons of dichotomous and categorical variables, the unpaired *t*-test for unadjusted comparisons of continuous outcomes. Multiple logistic regression analysis was employed to identify predictors of the presence of BM micrometastases. The level of statistical significance was set at .05. All statistical tests were two-sided. For compilation of data Microsoft Access database software (Microsoft Corporation, Redmond, WA, USA) was used. Statistical analyses were performed with SPSS software (Version 13.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Four hundred and ten patients underwent SLN biopsies together with BM aspirations. There were no statistically significant differences in the baseline characteristics of the patients with and without BM micrometastases (Table 1).

BM micrometastases were detected in 28.8% (118/410) of the patients. In the overall sample, 51.2% (210/410) of patients were SLN negative/BM negative, whereas 12.4% (51/410) were SLN positive/BM positive. Of all patients, 16.4% (67/410) of our sample were SLN negative/BM positive and 20.0% (82/410) SLN positive/BM negative (Table 2).

Patient Subset without Bone Marrow Micrometastases

In the subgroup of patients without BM micrometastases (n = 292), 71.9% (210/292) were also SLN negative, including 12 patients with isolated tumor cells. In this subset, SLN macrometastases were detected in 18.5% (54/292) and SLN micrometastases in 9.6% (28/292).

Patient Subset with Bone Marrow Micrometastases

In the subset of patients with BM micrometastases (n = 118), the SLN showed macrometastases in 25.4% (30/118) and micrometastases in 17.8% (21/118). In this subset, the SLN was negative in 56.8% (67/118), including two patients with isolated tumor cells.

Sentinel Lymph Node Status

The SLN were positive in 32.4% (133/410) of all patients. In six patients SLN micrometastases were

TABLE 1. *Baseline characteristics (n = 410 patients)*

	Sentinel lymph node					Bone marrow micrometastases				
	Negative (*n* = 277)		Positive (*n* = 133)			Negative (n = 292)		Positive (n = 118)		
Parameter	n	%	n	%	*P*-value	n	%	n	%	*P*-value
Age (years)										
Median	59.2		60.6		.947	59.2		60.4		.883
Range	28.4–85.1		28.3–82.4			28.3–85.1		31.1–82.2		
Tumor size (mm)										
Median	15.0		18.0		< .001	16		16		.577
Range	3–30		3–30			3–30		3–30		
T stage										
pT1a	8	29	1	0.8	.002	6	2.1	3	2.5	.195
pT1b	39	14.1	10	7.5		39	13.3	10	8.5	
pT1c	171	61.7	72	54.1		164	56.2	79	67.0	
pT2 ≤3 cm	59	21.3	50	37.6		83	28.4	26	22.0	
Quadrant										
Upper outer	162	58.5	64	48.1	.238	161	55.2	65	55.1	.712
Upper inner	44	15.9	31	23.3		57	19.5	18	15.2	
Lower outer	34	12.3	15	11.3		33	11.3	16	13.6	
Lower inner	20	7.2	13	9.8		24	8.2	9	7.6	
Central	17	6.1	10	7.5		17	5.8	10	8.5	
Histology										
Ductal	240	86.7	119	89.5	.474	253	86.6	105	89.0	.800
Lobular	27	9.7	12	9.0		29	10.0	10	8.5	
Others	10	3.6	2	1.5		10	3.4	3	2.5	
Primary tumor										
Tumorectomy	259	93.5	112	84.2	.004	267	91.4	104	88.1	.352
Mastectomy	18	6.5	21	15.8		25	8.6	14	11.9	
Grading										
G1	65	23.5	24	18.1	.332	68	23.3	21	17.8	.432
G2	153	55.2	74	55.6		160	54.8	67	56.8	
G3	59	21.3	35	26.3		64	21.9	30	25.4	
Number of SLN										
Median	2.0		2.0		.077	2.0		2.0		.322
Range	1–14		1–7			1–14		1–7		
Menopausal status										
Premeno	78	28.2	36	27.1	.906	210	71.9	85	72.0	1.000
Postmeno	199	71.8	97	72.9		82	28.1	33	28.0	
Estrogen receptor										
Positive	229	82.7	118	88.7	.143	245	83.9	102	86.4	.550
Negative	48	17.3	15	11.3		47	16.1	16	13.6	
Progesterone receptor										
Positive	201	72.6	109	81.9	.05	217	74.3	93	78.8	.375
Negative	76	27.4	24	18.1		75	25.7	25	21.2	

TABLE 2. *Association between SLN status and presence of BM micrometastases*

	BM negative	BM positive	Total
SLN negative	210 (51.2%)	67 (16.4%)	277 (67.6%)
SLN positive	82 (20.0%)	51 (12.4%)	133 (32.4%)
Total	292 (71.2%)	118 (28.8%)	410 (100.0%)

P-value: .004 (Fisher's exact test).
BM: bone marrow.

already detected by frozen section. The thorough examination of the SLN biopsies with step sectioning and immunohistochemistry for definitive histopathology revealed micrometastases in 43 patients and macrometastases in another three patients. This led to an upstaging effect of node negative to node positive patients in 16.6% (46/277) in our study. As expected, there were statistically significant differences between the subgroups of patients with negative and positive SLN in terms of tumor size ($P < .001$), tumor stage ($P = .002$), and the percentage of performed breast conserving therapy ($P = .004$) (Table 1).

Association between SLN Status and the Presence of Bone Marrow Micrometastases

There was a statistically significant association between the SLN and BM micrometastases status ($P = .004$). In multiple logistic regression analysis, the

TABLE 3. *Results of multiple logistic regression analysis*

	Presence of bone marrow micrometastases		
Variable	*P*-value	Odds ratio	95% CI
SLN positive versus negative	.007	1.860	1.181–2.929
Tumor size >1 cm versus ≤1 cm	.438	1.306	0.665–2.563
Grade 3 versus 1 and 2	.411	1.279	0.712–2.297
Postmenopausal versus premenopausal	.955	0.986	0.605–1.606
ER positive versus negative	.859	1.086	0.437–2.697
PR positive versus negative	.585	1.234	0.580–2.624

ER estrogen receptor.
PR progesterone receptor.

SLN status was the only significant predictor of BM micrometastases (Table 3). Tumor stage (pT1 and pT2 ≤3 cm), age, grading, estrogen and progesterone receptor status were not significantly associated with the presence of BM micrometastases (Table 3).

DISCUSSION

The present prospective multicenter investigation—the first one in the literature—provides compelling evidence that there is a significant association between positive SLN status and the presence of BM micrometastases in early stage breast cancer patients.

Immunocytochemistry, immunomagnetic bead enrichment, RT-PCR, flow cytometry, fluorescence in situ hybridization are the methods currently used to search for BM micrometastases.[16–18] This renders the comparison of study results in the literature difficult. Most of the above-mentioned methods are based on the detection of epithelial markers on the membrane or the cytoplasm of the tumor cells and in the majority of published investigations cytokeratin antibodies, epithelial membrane antigen (EMA), or tumor-associated glycoprotein-12 (TAG-12) were used.[19] Sufficient methodological validation of the detection antibodies has only been reported for cytokeratin antibodies. Braun et al.[2] analyzed BM samples from 191 patients without malignant disease and found cytokeratin-positive cells only in 1% (2/191).[2] The same monoclonal antibody A45-B/B3 binding to cytokeratin 8, 18, and 19 was also used in our study. Both normal and transformed epithelial cells do express these cytokeratins. Therefore, to minimize the risk of false positive results, all specimens were critically reviewed by our pathologist and malignant cells were identified based upon clearly defined morphological criteria of cancer cells.

The incidence of BM micrometastases in breast cancer patients varies considerably in the literature. The positivity ranges from 14 to 48%. This fluctuation is due to variations in stage distribution, as most studies included breast cancer patients with a stage I–III. Furthermore, there are differences in the expression of the target antigen sensitivity and specificity of the antibodies used, and patient series.[2,5–7,20–25]

Different follow-up studies showed a clear association between the presence of BM micrometastases and poor prognosis.[23] In fact, the presence of BM micrometastases was associated with a significant reduction in both distant disease-free and overall survival. The effect of BM micrometastases was especially impressive among node negative breast cancer patients. Their overall survival was similar to that of patients being node positive but without BM micrometastases.[2] Diel et al.[7] reported that BM micrometastases were an independent prognostic indicator for disease-free and overall survival. Other studies found a shorter relapse-free and overall survival for BM micrometastases positive breast cancer patients, however, in these investigations BM micrometastases were not an independent prognostic factor.[5,20] It is noteworthy that most of the published studies showing a significant difference in disease-free and overall survival had either a follow-up period below a median of 5 years[2,7] or could prove such differences only for the first intermediate follow-up phase, which subsequently disappeared for longer follow-up periods.[5,23] For longer follow-ups, the prognostic impact of BM micrometastases seems to become less important compared to the effect within the first 5 years post-surgery.[5,23] Gebauer et al.[21] found that tumor size and lymph node status were clearly stronger prognostic factors although BM micrometastases status was found to be an independent prognostic factor. Patients without axillary lymph node involvement, however, showed no difference in disease-free or overall survival regardless of the BM status.

A significant association between axillary lymph node involvement, tumor size, and the presence of BM micrometastases was found in different studies,[5,7,23] while other did not find such a correlation,

probably due to a low number of patients.[20,22] All these studies were based on the findings of a standard histopathologic examination: the axillary lymph nodes were bisected and stained with H&E. The only (retrospective) study analyzing the association of SLN metastases and BM micrometastases did not find any significant correlation.[26] In the above-mentioned study, isolated tumor cells in the SLN were also regarded as positive nodes. Furthermore, only three sections were performed on the SLN.

The present investigation is the first prospective study in the literature to analyze the association between the SLN status and the presence of BM micrometastases in breast cancer patients. We found a statistically significant association between positive SLN status and the presence of BM micrometastases in both bivariate as well as multivariable analysis. Nonetheless, the percentage of non-concordance (SLN negative/BM positive and SLN positive/BM negative) was considerable. It remains to be evaluated whether or not BM micrometastases do have a prognostic impact in our patient sample and if the prognostic value of BM micrometastases is independent from the SLN status.

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