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The detection of circulating tumour cells in blood of metastatic breast cancer patients is of prognostic relevance

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Purpose: The prognostic relevance of circulating tumour cells (CTC) in blood of metastatic breast cancer patients was evaluated and compared with established prognostic criteria.

Patients and methods: Blood samples from 119 breast cancer patient were examined in a retrospective analysis. For the detection of CTC in blood, a nested RT-PCR assay for mammaglobin mRNA was applied.

Results: In 42/119 (35%) patients CTC were detected. Patients with CTC positive blood samples at the time of diagnosis of metastases lived significantly shorter (median 18 months) than CTC-negative patients (median 51 months), suggesting that CTC serve as an additional prognostic parameter. CTC in blood were an independent prognostic parameter, associated with the highest risk of death compared to other risk factors examined (HR: 2.9). In addition, the appearance of CTC in blood of metastatic patients during treatment indicated poor prognosis. CTC-positive patients had a significantly shorter survival compared to patients remaining CTC negative during followup ($p < 0.01$).

Conclusions: CTC at the time of diagnosis of metastases are an independent prognostic factor for overall survival. During therapy, the detection of CTC is predictive for a shorter survival of patients with metastatic breast cancer.

Keywords: Circulating tumour cells, mammaglobin, breast cancer, prognosis, relevance

Introduction

In breast cancer patients, metastases can remain latent and undetectable for many years. The dissemination of aggressive tumour cells can originate from the primary tumour, but metastases themselves are able to colonize other organs, too [1]. Hence, the detection of circulating tumour cells (CTC) in blood from metastatic breast cancer patients might be of clinical relevance.

In blood, CTC are detectable via immunocytochemistry (ICC) or molecular methods, mainly reverse transcriptase-polymerase chain reaction (RT-PCR). In both techniques the expression of specific genes (proteins for ICC and mRNA for RT-PCR) is used as marker for the presence of CTC. One of the most frequently used marker genes for CTC in breast cancer patients is mammaglobin (MGB1, SCGB2A2, UGB2), originally described in 1996 [2]. Mammaglobin is a member of the secretoglobin gene family, able to form homo- and hetero-dimers [3, 4]. The biological function of secretoglobins has remained elusive, a potential involvement of these proteins in tumour biology has been assumed [5].

Mammaglobin is not only mainly expressed in female breast tissue and breast tumours, but also in a small proportion of salivary gland tumours and endometrial cancers [6]. The protein exists in two main forms (18 and 25 kDa) [7]. In breast tumours, the highest mammaglobin expression is associated with a less aggressive tumour phenotype, which is characterized by the expression of oestrogen or progesterone receptors, low nuclear grading, the absence of nodal invasion and with postmenopausal status of the patients. In addition, mammaglobin expression does not correlate with HER2 gene amplification or mutant p53 expression [6, 8–10].

Due to different methods used and marker genes applied, varying results concerning the frequency of CTC in blood were reported [11, 12]. Therefore, the clinical utility of CTC as prognostic factor is still under debate. We have shown earlier that mammaglobin positivity in blood represents evidence of CTC [13]. The objective of this study now is to elucidate the prognostic relevance of detecting mammaglobin mRNA by RT-PCR as marker for CTC in blood of metastatic breast cancer patients. We will demonstrate that CTC were detected in more than one-third of these patients and they lived significantly shorter than CTC-negative patients. Moreover, the occurrence of CTC during treatment of metastatic patients indicates a shorter survival compared to those continuously negative for CTC.

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Patients and methods

Patients

Blood samples from 119 metastatic breast cancer patients (29–84 years, mean 61 years; 36 primary metastatic, 83 relapses) were tested after informed consent according to local legacy of the hospital was taken. The median followup of all patients was 2 years.

Within a period of 9 years, 93 of 119 metastatic patients were repeatedly tested along the course of their disease while the remaining 26 patients refused adequate treatment or were treated at other institutions further on. Sixty of these patients were CTC negative in repeated tests during treatment even though 13 of them were CTC positive at diagnosis of metastasis. The remaining 33 patients had recurring CTC positive tests.

Samples were taken at the physician decision from various departments of the hospital without proposed time scheduling. Patients were treated according to the commonly established regimen for metastatic breast cancer patients. Choice of therapy was not affected by CTC results in this retrospective study. Physicians as well as caregivers were blinded to CTC results.

Assay methods

Ten mililiter of peripheral blood per patient was taken and after lysis of erythrocytes total RNA from nucleated cells was prepared. All samples were processed within six hours after collection with an interim storage at 4°C. A nested RT-PCR assay for mammaglobin mRNA was performed in quadruplicates as described earlier [13]. Samples were assessed as positive if at least three PCR setups were positive [14]. In each assay, RNA prepared from breast-cancer cell-lines and from blood of healthy individuals was used as positive and negative controls, respectively.

Study design

In this retrospective study, breast cancer patients with metastatic disease diagnosed between 1997 and 2006 at a single institution were included. On average, six samples per patient at different time points were tested. As clinical endpoint overall survival was examined, variables for prognostic analysis are listed in Tab. 1.

Tab. 1: Prognostic characteristics of 119 metastatic breast cancer patients tested for mammaglobin expression in blood at the time of diagnosis of metastases

Factor	Result	N	%
Mammaglobin	positive	42	35
	negative	77	65
Age at metastasis*	<50 years	31	26
	>50 years	88	74
Time of metastasis*	Dx	36	30
	<2 years	24	20
	>2 years	59	50
Oestrogen receptor*	positive	84	71
	negative	33	28
	n.a.	2	1
Localization*	bone only	26	22
	visceral	93	78
Therapy	no chemotherapy	28	23
	any chemotherapy	91	77

n.a.: Not available
Time of metastasis: metastasis at diagnosis of breast cancer (Dx), relapse with distant metastases within or after 2 years from diagnosis of breast cancer (<2a, >2a)
Localization: metastases in bone only or in any other organ (visceral)
No chemotherapy: hormones or none
* Prognostic parameters for metastatic breast cancer patients according to [15]

Statistical analysis

Kaplan–Meier survival curve and the log-rank test were used to determine the univariate significance of risk factors. To examine the effects of multiple covariates on survival, Cox proportional-hazards regression model was applied. CTC togeth-

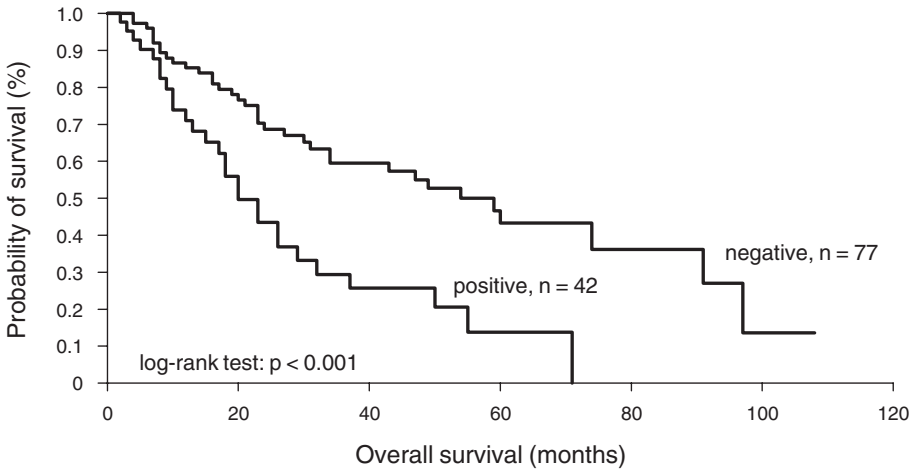

Fig. 1: Kaplan–Meier survival curves of 119 breast cancer patients tested for CTC in blood at the time of diagnosis of metastases

er with other prognostic criteria for metastatic breast cancer patients (oestrogen-receptor expression, time of metastasis, localization of metastases, age and chemotherapy [15]) were regarded for statistical analysis.

Results

Blood samples from 119 breast cancer patients with metastatic disease were tested for mammaglobin expression at the time of diagnosis of metastasis, 42 (35%) of them were positive. The incidence of CTC was similiar in primarily metastatic patients (stage IV) and in patients at relapse: 13/36 (36%) and 29/83 (35%), respectively.

During an overall observation period of 9 years, 28/42 (67%) mammaglobin positive patients died within 2–71 months (median 18 months) due to disease progression, compared to 36/77 (47%) CTC-negative patients (4–97 months, median 51 months) (Fig. 1). This difference is statistically significant ($p < 0.01$).

The distribution of prognostic factors relevant for patients with metastatic breast cancer [15] is shown in Tab. 1. In a multivariate analysis, only mammaglobin expression in blood was found to correlate significantly with overall survival ($p < 0.01$) (Tab. 2). Patients who were CTC positive at the time of diagnosis of metastases had a 2.9-fold increased risk to die compared to CTC-negative patients. In this small group of patients, the association of survival with oestrogen-receptor expression, time of metastasis (at diagnosis, relapse within or after 2 years from diagnosis of breast cancer), localization of metastases, age and chemotherapy did not reach significance, although tendencies to their prognostic value were seen (Tab. 2).

During followup 33 CTC-positive patients were either positive in all samples from the first appearance of their metastatic disease (10 patients) or became positive (23 patients) after a median observation period of CTC negativity of 13 months (2–91 months). Twenty-eight (85%) of them died at a median survival time of 10 months (1–35 months) after the detection of the first mammaglobin positive sample. On the other hand, 21 of 60 patients (35%) remaining persistently CTC negative died at a median time of 58 months (2–108 months) after diagnosis of metastatic disease (Fig. 2).

Tab. 2: Multivariate Cox regression of commonly used prognostic factors for overall survival of metastatic breast cancer patients [15]

Factor	p value univariate	Hazard ratio (95% CI)	p value multivariate
Mammaglobin negative vs. positive	0.0003	2.9491 (1.7306–5.0258)	0.0001
Age at metastasis <50 vs. >50	0.1423	0.5644 (0.3159–1.0085)	0.0534
Time of metastasis Dx vs. <2a vs. >2a	0.1662	1.4413 (0.7231–2.8725)	0.3287
Oestrogen receptor positive vs. negative	0.3640	0.6714 (0.3582–1.2585)	0.2139
Localization bone vs. visceral	0.1826	0.7452 (0.3904–1.4224)	0.3726
Therapy chemotherapy vs. other	0.5031	0.8822 (0.4638–1.6780)	0.7024

Discussion

Due to the low number of CTC in patients with solid tumours detection techniques of particular sensitivity are needed for clinical application [16]. The mammaglobin nested RT-PCR assay has been shown to be one of the most sensitive as well as specific test for the detection of CTC in blood from breast cancer patients [13, 14]. For this retrospective analysis we have chosen this method to evaluate the prognostic relevance of CTC in metastatic breast cancer patients.

Applying nested RT-PCR, we were able to detect mammaglobin mRNA transcripts in all 52 breast-tumour samples analyzed [17], irrespectively from grading, hormone-recep-

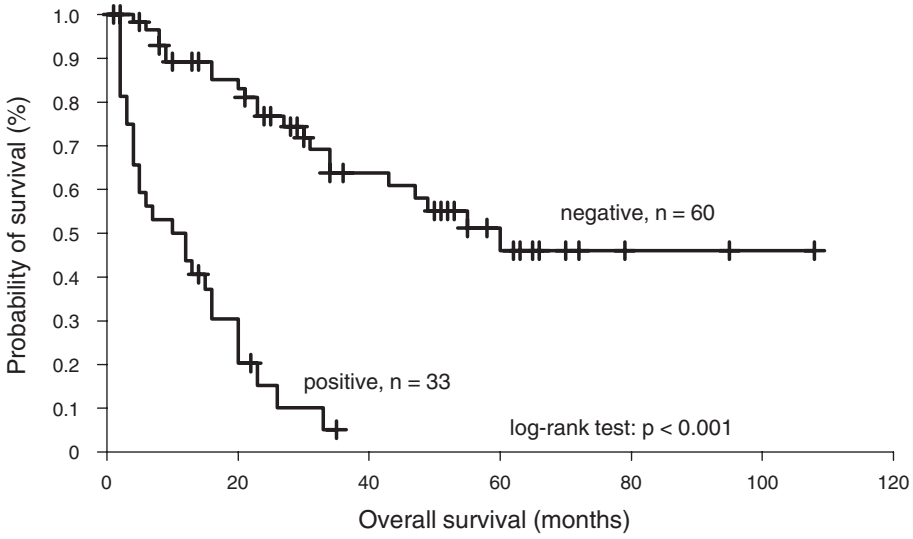

Fig. 2: Kaplan–Meier survival curves of metastatic patients tested repeatedly CTC negative or at least once CTC positive during treatment

tor expression or lymph-node involvement. Apparently, all breast carcinomas express mammaglobin although quantitative measurement of mammaglobin mRNA in the primary tumour revealed large variability. Low expression was associated with a less aggressive phenotype [6, 8–10]. On account of its remarkable specificity for cells derived from breast tissue mammaglobin is a reliable marker for CTC detection in blood from breast cancer patients, irrespectively of the phenotype of the primary tumour.

Various molecular markers for the detection of CTC are in use [11, 12, 16]. The main problem for finding an optimal marker is the occurrence of false-positive results due to illegitimate expression of the marker genes detected by highly sensitive molecular methods. Therefore thresholds have to be introduced into each specific assay. Concerning the assay applied in this study, four PCR setups per samples were routinely carried out. To define a threshold, samples from 143 patients with benign lesions of the breast were tested [14], 4% of them were mammaglobin positive in one or two PCR setups. Because none of them was positive in three or four PCR setups, a CTC positive blood sample was defined as mammaglobin mRNA positive in at least three PCR setups [14].

In previous reports the incidence of CTC in blood of metastatic breast cancer patients ranged from 25 to 73% [11], reaching variable prognostic relevance for these patients. This difference may depend in most circumstances on the method used to detect CTC among several other factors. In the small cohort of the study presented here, more than one-third of patients were mammaglobin positive. These patients lived significantly shorter than CTC-negative patients (Fig. 1). Compared with other well-known risk factors examined, the occurrence of CTC in blood samples was found to be the most reliable marker for prediction of a poor overall survival (Tab. 2). Hence, at the time of diagnosis of metastases the appearance of CTC in blood should be determined in these patients due to its prognostic value [18].

In addition, monitoring of patients during treatment seems to be of clinical relevance for assessment of response to therapy. Metastatic patients who persist CTC negative have a significantly longer overall survival than metastatic patients who become CTC positive during treatment (Fig. 2). This observation confirms earlier reports that changes in CTC levels during therapy are associated with the risk of progression in metastatic breast cancer patients [19].

Regarding the methods used it is obvious that CTC in blood of non-metastatic breast cancer patients yield conflicting results [11]. However, it should be noted that our mammaglobin RT-PCR assay reveals a CTC-detection rate of only 3% in these breast cancer patients (stages I–III) (data not shown). The detection rate of CTC might be increased when introducing additional markers to the assay, but it is questionable whether these markers exhibit comparable specificity for cells derived from breast tissue as demonstrated for mammaglobin.

In conclusion, our data provide evidence to suggest that at the time when metastatic disease is clinically diagnosed, CTC are an independent prognostic parameter for overall survival. During treatment of metastatic disease the occurrence of CTC in blood predicts an inferior outcome for these patients. We recommend the mammaglobin nested RT-PCR assay for the detection of CTC in blood from patients with metastatic disease and during followup of these patients.

This manuscript was prepared according to the REMARK criteria for tumour marker studies [20].

Take-home message

Mammaglobin is a reliable marker for the detection of CTC in blood from breast cancer patients. In metastatic patients CTC are a prognostic parameter for overall survival.

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References

[1] Nguyen DX, Massague J. Genetic determinants of cancer metastasis. Nat Rev Genet, 8: 341–352, 2007.
[2] Watson MA, Fleming TP. Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res, 56: 860–865, 1996.
[3] Sjoedin A, Guo D, Lund-Johansen M, et al. Secretoglobins in the human pituitary: high expression of lipophilin B and its down-regulation in pituitary adenomas. Acta Neuropathol, 109: 381–386, 2005.
[4] Carter D, Douglass JF, Cornellison CD, et al. Purification and characterization of the mammaglobin/lipophilin B complex, a promising diagnostic marker for breast cancer. Biochemistry, 41: 6714–6722, 2002.
[5] Sjödin A. Human secretoglobins in normal and neoplastic cells and tissues. *http://urn.kb.se/resolve?urn=urn:nbn:se:umu:diva-490*, 2005.
[6] Sasaki E, Tsunoda N, Hatanaka Y, et al. Breast-specific expression of MGB1/mammaglobin: an examination of 480 tumors from various organs and clinicopathological analysis of MGB1-positive breast cancers. Mod Pathol, 20: 208–214, 2007.
[7] O'Brien NA, O'Donovan N, Ryan B, et al. Mammaglobin a in breast cancer: existence of multiple molecular forms. Int J Cancer, 114: 623–627, 2005.
[8] Culleton J, O'Brien N, Ryan BM, et al. Lipophilin B: a gene preferentially expressed in breast tissue and upregulated in breast cancer. Int J Cancer, 120: 1087–1092, 2007.
[9] Span PN, Waanders E, Manders P, et al. Mammaglobin is associated with low-grade, steroid receptor-positive breast tumors from postmenopausal patients, and has independent value for relapse-free survival time. J Clin Oncol, 22: 691–698, 2004.
[10] Nunez-Villar MJ, Martinez-Arribas F, Pollan M, et al. Elevated mammaglobin (h-MAM) expression in breast cancer is associated with clinical and biological features defining a less aggressive tumor phenotype. Breast Cancer Res, 5: R65–R70, 2003.
[11] Zach O, Lutz D. Tumor cell detection in peripheral blood and bone marrow. Curr Opin Oncol, 18: 48–56, 2006.
[12] Lacroix M. Significance, detection and markers of disseminated breast cancer cells. End Rel Cancer, 13: 1033–1067, 2006.
[13] Zach O, Kasparu H, Krieger O, et al. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. J Clin Oncol, 17: 2015–2019, 1999.
[14] Zach O, Wagner H, Kasparu H, et al. Statistical validation of the mammaglobin-nested RT-PCR assay for tumor cell detection in blood of breast cancer patients. BioTechniques, 31: 1358–1362, 2001.
[15] Schmid P, Wischnewsky M, Sezer O, et al. Prognostic factors are more important for overall survival (OS) than treatment outcome in patients with metastatic breast cancer (MBC). Ann Oncol, 11(Suppl 4): 27, 2000.
[16] Alix-Panabieres C, Müller V, Pantel K. Current status in human breast cancer micrometastasis. Curr Opin Oncol, 19: 558–563, 2007.
[17] Zach O, Lutz D. Mammaglobin remains a useful marker for the detection of breast cancer cells in peripheral blood. J Clin Oncol, 23: 3160, 2005.

[18] Bidard FC, Vincent-Salomon A, Sigal-Zafrani B, et al. Prognosis of women with stage IV breast cancer depends on detection of circulating tumor Cells rather than disseminated tumor cells. Ann Oncol, 19: 496–500, 2008.
[19] Nole F, Munzone E, Zorzino L, et al. Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications. Ann Oncol, 19: 891–897, 2008.
[20] McShane LM, Altman DG, Sauerbrei W, et al. Statistics Subcommittee of NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumour MARKer prognostic studies (REMARK). Breast Cancer Res Treat, 100: 229–235, 2006.