ORIGINAL ARTICLE

BONE MARROW MICRO-METASTASES IN CLINICALLY STAGE I-III HER2/NEU POSITIVE BREAST CANCER

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Aim: *Patients with breast cancer should have a favorable outcome when diagnosed early. Occult distant metastases, especially in the Bone Marrow (BM) was claimed to be responsible for tumor relapse in certain cases, especially in HER-2/neu expressing tumors. Such an occult metastases may be detected by PCR techniques for a BM aspirate, which is the aim of this study.*
Methods: *Between May 2004 and May 2005, PCR technique was used to determine the tumor HER-2/neu status of 37 patients, attending Suez Canal University Hospital for the treatment of stage I-III breast cancer. BM and peripheral blood of those positive patients were examined by the same technique for the presence of micro-metastases. Correlation between tumor characteristics and the HER-2/neu status was done.*
Results: *Fifteen patients were tumor HER-2/neu positive, which correlated with stage and number of positive lymph nodes. BM micro-metastases were detected in 7 patients, with no correlation to the tumor characteristics. Additionally, HER-2/neu positive tumor cells were detected in the peripheral blood of 6 patients.*
Conclusion: *BM micro-metastases can be detected in HER-2/neu positive non-metastatic breast cancer that may be predicted by the peripheral blood not the primary tumor characteristics.*

Keywords: *Disseminated tumor cells, Occult metastases, C-erbB2.*

INTRODUCTION

Breast cancer is a common problem with obvious morbidity and mortality.(1) It has been recognized that late presentation of patients with breast cancer carries the worst prognosis.(2) Therefore, screening programs have been applied to detect malignant lesions as early as possible.(3) This facilitate the ability not only to carry a curative operation, but also a conservative one as well.(4,5)

In spite of this progress in management, metastatic disease appears within 5 years in 30-50% of the patients who had what thought to be a definitive treatment.(1,6,7) This may suggest that cancer in these patients has already spread at the time of presentation(5,7-9) that was missed by the conventional screening tools because it was micro rather than macro-metastases.(10) Therefore, the need for additional tools to diagnose tumor dissemination has become an issue of interest.

Since the BM is considered to mirror the efficacy of the metastatic process throughout the body(1) and its loading with tumor cells considered to be an independent prognostic factor,(9,11) it became the site of interest to detect occult metastases, suggesting that this minimal disease is indeed the progenitor of manifest metastasis.(12,13) Fortunately, the detection of micro-metastases became possible by the development of monoclonal antibodies to

epithelial cytokeratins and tumor-associated cell membrane glycoproteins (Oncogene).[7]

An important member of the oncogene family is the Human Epidermal growth factor Receptor-2 (HER-2/neu).[14,15] It is over-expressed in 15-30% of invasive breast cancer and associated with metastatic relapse and poor prognosis.[3,10] HER2/neu over-expressing tumors has a specific "biological" therapy (Herceptin) that attacks the disseminated tumor cells with less systemic side effects.[1] Furthermore, a specific vaccine is under development that may change the future management of such tumors.[16]

The most widely used methods for measuring HER-2/neu are immuno-cytochemistry (ICC), Fluorescent in situ hyperdization (FISH) and Enzyme linked immunsorbent assay (ELISA). However, both ICC and FISH are complex and subject to technical errors. Moreover, FISH does not assess gene expression and cannot identify cases in which the gene product is over-expressed in the absence of gene amplification.[6] On the other hand, Polymerase chain reaction (PCR) based assays are currently the most sensitive method for the detection of micro-metastatic disease, it can detect one tumor cell in the back ground of 107 bone marrow cells.[12]

More recently, circulating tumor cells in the serum of non metastatic breast cancer patients were detected. Their presence was associated with relapse and distant organ affection. This new field is gaining interest due to the simplicity of the blood sample in comparison to the BM.[7]

Therefore, in this study PCR technique was used to detect Bone marrow micro-metastases in HER2/neu positive breast cancer patients, in whom the tumor assumed to be loco-regional (stage I-III). Additionally, the peripheral blood of those patients with HER-2/neu positive primary tumor, was tested for the presence of circulating HER-2/neu positive tumor cells.

PATIENTS AND METHODS

A prospectively designed study was conducted in the period between May 2004 and May 2005. Female patients of any age, admitted to the Suez Canal University Hospital - Egypt, diagnosed to have breast cancer using the triple assessment according to the diagnostic guidelines,[13,17] were included in this study.

All patients were investigated for the presence of metastases and accordingly staged (according to the TNM classification)[18] by chest X-ray, Liver ultra-sound scan, bone isotope scan, liver enzymes and serum calcium.[1] Patients with the following criteria were excluded from this study:

- Distant organ metastases (Stage IV).
- Patients unfit for surgery.

Out of 70 patients diagnosed to have breast cancer during the study period, we were able to recruit 37 eligible patients for the study. All signed an informed consent, and the Departmental Council approved the study medically and ethically.

Surgical and sampling procedure: Patients had either Modified Radical Mastectomy with axillary clearance or simple mastectomy according to their stage.[1,2] During the operation, bone marrow aspiration was performed from the sternum. This was done through the medial end of the surgical wound to obtain 5 ml of bone marrow. In addition, two more 5 ml of bone marrow samples were obtained from each anterior iliac crest. Samples obtained were pooled and immediately put into tubes containing Ethyl diamine tetra-acetic acid (EDTA) to prevent clotting. Bone marrow samples were sent to the hospital lab together with a sample of the breast tissue tumor and a venous blood sample collected on EDTA tubes. The rest of the resected breast and axillary tissue was sent to the Pathological Laboratory.

Laboratory procedure: The sample of the breast tissue tumor was put in a clean container then flash frozen in liquid nitrogen for further RNA purification. BM and blood samples were stored by adding 1ml of anticoagulated bone marrow/blood sample to 5 ml preservative (RNA/DNA stabilizing reagent for blood/bone marrow from Roche molecular diagnostics) and were frozen at -20 for further RNA purification.

From the pathological laboratory, data regarding the tumor histomorphology, tumor-node staging, grading and results of the BM microscopic examination for disseminated metastatic cells were collected for all patients.

In the clinical pathology laboratory, the samples of the breast tissue tumor were examined by the PCR technique for HER2/neu over expression for all patients. Due to financial reasons, only positive patients had their bone marrow and peripheral blood examined for the presence of disseminated HER-2/neu over-expressing cells.

Tissue samples were disrupted and homogenized by cryosectioninig the frozen tissue, then about 350 microns of the lysis-binding buffer were added, after which tissue samples were manually homogenized. BM and peripheral blood samples were pre-treated before RNA purification by pre-warming lysates at 15-25 ^{o}C. RNA extraction was carried out using the MagNA Pure LC RNA Isolation Kit for Blood/bone marrow from Roche molecular diagnostics, on the MagNA pure Light Cycler (LC) instrument.

Analysis of HER-2/neu mRNA was carried out using the LC HER-2/neu mRNA quantification kit from Roche

molecular diagnostics. The procedure was explained in the attached manual. Quantification of RNA encoding for HER-2/neu mRNA was done in two steps; Reverse transcription, that include complementary DNA synthesis followed by PCR amplification technique. During this technique a395 bp fragment of HER-2/neu mRNA was amplified from the cDNA by PCR using specific primers (ready to use primer and hyperdisation probe mixture specific for human HER-2/neu mRNA, from Roche Molecular Biochemicales). The amplicon was then detected by fluorescence using a specific pair of hyperdization probes. The hyperdization probes consist of two different oligonucleotides that hyperdize to an internal sequence of the amplified fragment during the Annealing phase of the amplification cycle. One probe is labeled at the 5'-end with light cycler Red 640, and to avoid extension, modified at 3'-end by phosphorylation. The other probe was labeled at the 3' end with light cycler flourescin. Only after hyperdization to the template DNA, the two probes come in close proximity, resulting in Fluorescence resonance energy transfer (FRET) between the two flourophores. The emitted fluorescence of light cycler red 640 is then measured by the light cycler instrument.

Test data analysis was performed by using the relative quantification software for the light cycler instrument, the amount of mRNA encoding for HER-2/neu was expressed as a relative ratio to a reference gene (G6PDH) in a sample relative to the HER-2/neu / G6PDH ratio in a calibrator. Results were interpreted by calculating the amount of target HER-2/neu as a ratio of target (T) gene copies to reference gene copies (G6PDH). In the second step, the ratio of the target to reference copies (T:R) in the sample is divided by the T:R ratio in the calibrator the is run with each sample reaction in parallel

Patients with positive HER2/neu results were informed and transferred to the oncology department for further management.

Data and statistical analysis: Statistical analysis was conducted by calculating simple means and standard deviations for continuous variables, and frequency counts and percentages for categorical variables. Student's t test or qui square analysis/were used to assess the correlation between variables. Significance was set at $P<0.05$. All analysis was performed with the SPSS 13.0 for Windows 2003 software.

RESULTS

Over the period of 12 months, thirty-seven eligible female patients were recruited in the study. Their age ranged from 27 to 65 years with a mean of 47.3 ± 9.33 years. Four patients (10.8%) were in stage I, 28 patients (75.7%) were in stage II and 5 (13.5%) patients were in stage III.

Table 1 summarizes the positive HER-2/neu results. Using the PCR technique HER-2/neu was over expressed in breast tissue tumor of 15 patients (40.6%) of the whole sample. In addition, it was over expressed in the bone marrow of 7 patients (46.7%) and in the peripheral blood of 6 patients (40%) out of those 15 positive breast tissue tumor patients.

The characteristics of the breast tissue HER-2/neu positive and negative groups of patients are shown in Table 2.

The Breast tissue tumor was HER-2/neu positive in 39.3% of patients in stage II and in 80% of those in stage III. HER2/neu positive status correlated significantly with the tumor staging. It was also correlated significantly with the number of positive lymph nodes, as it was found in 11.1% of node negative patients, 46.1% of those with 1-3 positive lymph nodes and in 53.3% with>3 positive lymph nodes were.

The tumor size in the removed breasts ranged from 1.3-5.6 cm with a mean of 3.34 ± 1.3 cm. The mean tumor size in HER-2/neu positive and negative patients was nearly similar (3.36 and 3.32 cm respectively) with no statistical significance. Moreover, the correlation between the increase in tumor size and HER-2/neu positive status was statistically insignificant.

The primary tumor was positive for HER-2/neu in 25% of patients with grad I tumors. The frequency of positive HER-2/neu tumors increased as the pathological grad increased. However, this relation was statistically insignificant. We did not observe a statistical correlation between HER-2/neu positive status and the tumor type.

Bone marrow staining and microscopically examination for the whole studied population (n=37) did not detect any tumor cells. However, using the PCR technique, HER-2/neu positive tumor cells were detected in the BM of 7 patients (46.7%) out of the 15 HER-2/neu positive primary tumors. Figure (1) shows an example of BM and breast tissue positive result. The characteristics of these patients in relation to the BM HER-2/neu negative patients are summarized in Table 3.

Tumor cells expressing HER-2/neu were detected in the BM of 45.5% of patients in stage II, in the single patient with 0 lymph node and in different levels and types of the excised tumors. None of these studied tumor characteristics was observed to correlate significantly with the BM HER-2/neu positive status.

Lastly, HER-2/neu positive circulating tumor cells were detected in 6 patients (40%) out of the 15 HER-2/neu positive primary tumors. Those 6 patients were all BM HER-2/neuI positive, Table 3.

Table 1. Distribution of the positive results in relation to the total population.

	HER2/neu negative		HER2/neu positive	
	%	Number	%	Number
Breast tumor tissue	15/37	40.5	22/37	59.5
Bone Marrow	7/15	46.7	8/15	53.3
Peripheral Blood	6/15	40	9/15	60

Table 2. Distribution of the study population according to breast tumor HER-2/neu.

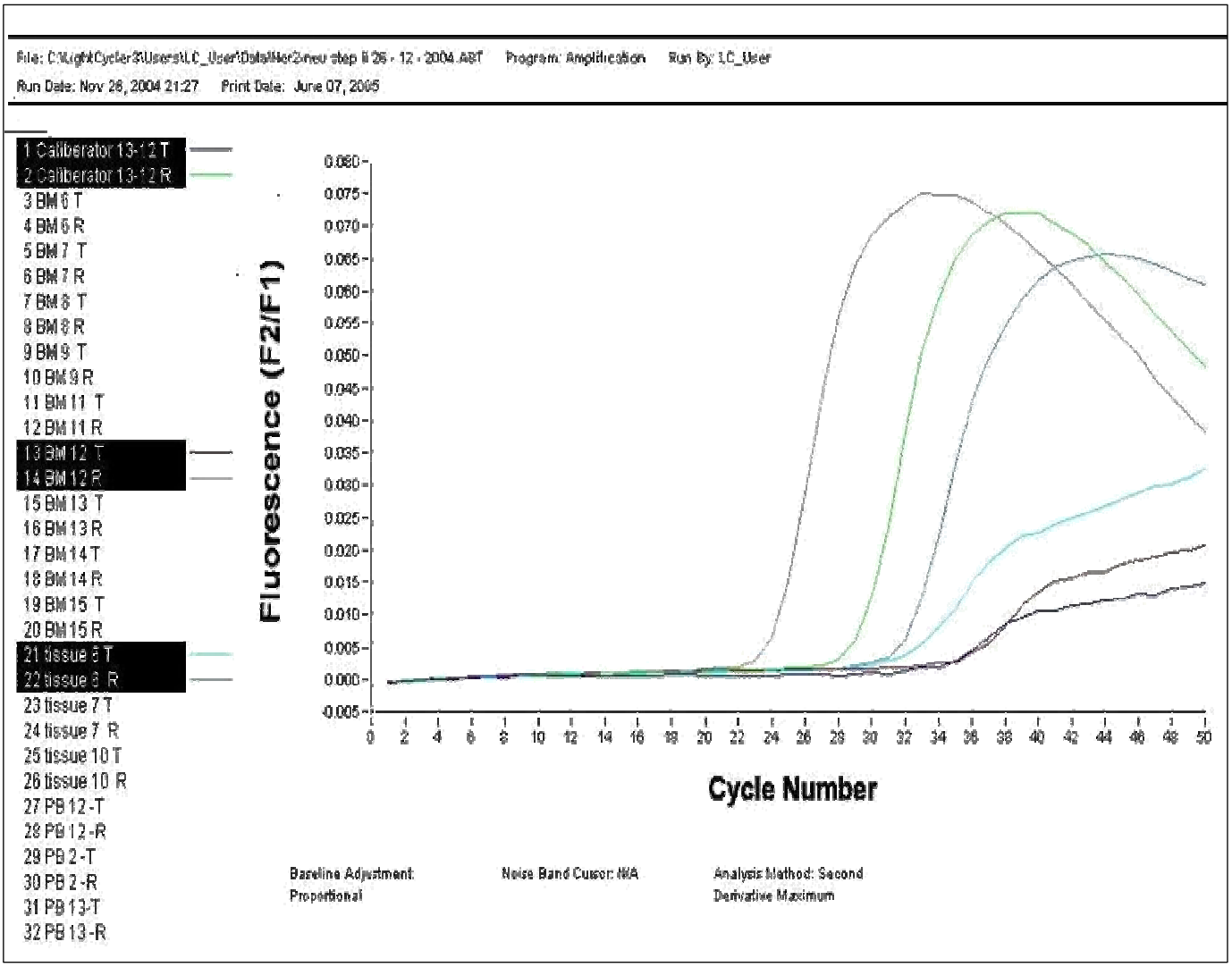
Variable		Breast tumor tissue HER-2/neu Number (%)		Total	P value
		Positive	Negative	37	
		N= 15 (40.6%)	N=22 (59.4%)		
Mean age ± std. deviation (years)		**49.07 ± 10.2**	**46.09 ± 8.7**	**-**	**0.285**
Stage	I	0 (0)	4 (100)	4(10.8)	0.024*
	II	11 (39.3)	17 (60.7)	28(75.7)	
	III	4 (80)	1 (20)	5(13.5)	
Histological LN state	0	1 (11.1)	8 (88.9)	9(24.3)	0.014*
	1-3	6 (46.1)	7 (53.8)	13(35.2)	
	>3	8 (53.3)	7 (46.7)	15(40.5)	
Tumor size	Mean± std.	3.36 ± 1.5 cm	3.32 ± 1.1 cm	-	0.942
	T1	3 (42.9)	4 (57.1)	7(18.9)	0.315
	T2	8 (34.8)	15 (65.2)	23(62.2)	
	T3	4 (57.1)	3 (42.9)	7(18.9)	
Grad	I	1 (25)	3 (75)	4(10.8)	0.481
	II	8 (40)	12 (60)	20(54)	
	III	6 (46.1)	7 (53.8)	13(35.2)	
Tumor Type	IDC	10 (41.6)	14 (58.3)	24(64.9)	0.917
	ILC	2 (28.6)	5 (71.4)	7(18.9)	
	Medullary	2 (66.7)	1 (33.3)	3(8.1)	
	Mucinous	1 (50)	1 (50)	2(5.4)	
	Papillary	0 (0)	1 (100)	1(2.7)	

- St. d.: Standard deviation.
- LN state: lymph node status.
- IDC: invasive duct carcinoma.
- ILC: invasive lobular carcinoma.
- *: P value < 0.05 is significant.

Table 3. Characteristics of Bone Marrow HER-2/neu positive patients.

Variable		BM HER-2/neu Number (%)		Total	P value
		Positive	Negative	15	
		N = 7 (46.7)	N= 8 (53.3)		
Mean age ± std. deviation (in years)		50.7 ± 9.2	47.6 ± 11.5	-	0.580
Stage	I	-	-	-	0.662
	II	5 (45.5)	6 (54.5)	11(73.3)	
	III	2 (50)	2 (50)	4(26.7)	
LN state	0	1 (100)	0 (0)	1(6.7)	0.553
	1-3	1 (16.7)	5 (83.3)	6(40)	
	>3	5 (62.5)	3 (37.5)	8(53.3)	
Tumor size	Mean ± std.	3.8 ±1.2 cm	3.1 ±1.5 cm	-	0.299
	T1	0 (0)*	3 (100)	3(20)	0.178
	T2	5 (62.5)	3 (37.5)	8(53.3)	
	T3	2 (60)	2 (40)	4(33.3)	
Grad	I	1 (100)	0 (0)	1(6.7)	0.780
	II	3 (39.5)	5	8(53.3)	
	III	3 (50)	3 (50)	6(40)	
Tumor type	IDC	4 (40)	6 (60)	10(66.7)	0.141
	ILC	0 (0)	2 (100)	2(13.3)	
	Medullary	2 (100)	0 (0)	2(13.3)	
	Mucinous	1 (100)	0 (0)	1(6.7)	
	Papillary	-	-	-	
Blood HER2/neu	Negative	1 (11.1)	8 (88.9)	9(60)	0.001*
	Positive	6 (100)	0 (0)	6(40)	

- St. d.: Standard deviation.
- LN state: lymph node status.
- IDC: invasive duct carcinoma.
- ILC: invasive lobular carcinoma.
- *: P value < 0.05 is significant.



- BM T: The targeted gene in the BM (HER-2/*neu*).
- BM R: The reference gene in the BM (G6PDH).
- Tissue T: The targeted gene in the tissue (HER-2/*neu*).
- Tissue R: The reference gene in the tissue (G6PDH).
- PB T: The targeted gene in the peripheral blood (HER-2/*neu*).
- PB R: The reference gene in the peripheral blood (G6PDH).
- Cycle Number: The number of the amplification cycles.
- Fluorescence: The amplitude of imitated fluorescence (light).

- N.B.: The figure shows the positive results (BM sample NO.13 and breast tissue sample NO. 21) in addition to their reference G6PDH (sample 14&22) and the test procedure calibrator (samples 1&2).

Fig 1. An example of positive BM & breast tissue samples.

DISCUSSION

The present study reports the detection of tumor cells in the BM of patients with HER-2/neu positive breast cancer, stage I-III, whom assumed by standard clinical and radiological methods to be non-metastatic.

Most of our patients were in stage I-II (86.5%) and only 13.5% of them were in stage III, which mean that most of our patients assumed to have a curative surgery. The finding of BM micro-metastases, particularly in these patients, is of great clinical impact on their plan of management.

In previous reports, ICC was used to determine the HER-2/neu status of the primary breast tumor. Bjorn et al. reported 7% positive results, such a low percentage was explained in the same report by a technical issue.[3] However, most reports suggests that HER-2/neu positive primary tumor range from 30 to 38% (3,10,20). In this study, the percentage was 40.6%, which agrees with the previous global figures.

We observed a significant correlation between HER-2/neu positive status and both the advances in clinical stage and the number of the affected lymph nodes. This correlation was suggested previously,[10] which may indicate that patient's stage and lymph node status have reflected the aggressive behavior of the HER-2/neu positive tumors.

On the other hand, one out of 9 patients with negative lymph nodes had HER-2/neu positive breast tumor. This observation is more convincing when previous reports, especially those included early-diagnosed patients with larger sample size, are considered. In these reports, HER-2/neu positive primary tumors were found in 16-30% of patients with negative lymph nodes.[7,10,21] Additionally, in the present study, 42.9% of tumors <2 cm in size were HER-2/neu positive. These findings may indicate that in spite of the tumor aggression, early diagnosis can pick up HER-2/neu positive tumors in the initial course of the disease. Therefore, patients with early breast cancer can neither be considered safe nor ignored for the suspicious of having HER-2/neu tumors.

All patients proved by clinical and radiological studies to have no BM metastasis, which was proved true by the ordinary microscopic examination of the BM samples. However, using the PCR technique, 46.7% (7 out of 15) HER-2/neu positive breast tissue patients showed metastatic cells in their BM. This agrees with Stephan et al, who by using ICC technique, reported 60% HER-2/neu positive BM micro-metastases in a selected sample of 52 patients, all have CK-18 positive BM and were stage I-III. His results had a very significant clinical impact, as 97% of these patients had clinically distant metastases after an observation period of 64 months.[10]

From another point of view, our seven BM HER-2/neu positive patients represent 18.9% of the whole studied population. Although, previous reports agrees about the presence of these cells in a non selected sample of non-metastatic breast cancer patients, however, the frequency have not been uniform, particularly when different diagnostic modalities were used.[14] Bjorn reported only 9.9% positive BM by using ICC technique, in the same article, this percentage increased to 16.1% when Immunomagnetic enrichment followed by ICC were used for the same samples.[3] In other reports ICC methods were able to detect BM micro-metastases in up to 29% of non-metastatic breast cancer patients.[8,21] Therefore, we believe that using the PCR technique is an advantage for this study and our results may be more accurate.

The finding of micro-metastases in apparently non-metastatic patients has a significant clinical impact, as up to recent, still a group of surgeons believes that early operable breast cancer (T1-2, N0-1) should have minimal metastatic work up (chest x-ray, blood count, and liver enzymes).[8] However, in this study, none of the studied tumor characteristics had a significant statistical correlation with the positive BM results. This agrees with previous reports, in which BM micro-metastases was proved by different techniques to be independent of any tumor characters.[3,8,10,21] Therefore, BM micro-metastases may be discovered irrespective of the tumor stage, lymph node status, tumor size, or grad. However, the correlation of other tumor characteristics, that were not included in this study such as vascular invasion and hormone receptor status, need to be determined.

Moreover, the correlation between BM micro-metastases and tumor relapse with distant metastases has been previously proved.[7,14] Therefore, all breast cancer patients with HER-2/neu positive tumor may be considered for BM examination for micro-metastases before the strategy of therapy decided.[22] This can be achieved by determination of the breast tissue HER-2/neu status in a tumor sample obtained through a preoperative diagnostic core biopsy.[20]

Simultaneous peripheral blood examination for the presence of circulating HER-2/neu positive cells was performed. Interestingly, this yields positive circulating tumor cells in the serum of 6 out of the same 7 BM positive patients. This might suggests that presence of circulating malignant cells reflects high possibility of BM micro-metastases. This important observation agrees with the recent studies demonstrating the presence of these circulating tumor cells in non-metastatic breast cancer patients, whom proved latter to have a poor outcome.[9,23] This interesting finding has another important clinical

impact; as the diagnosis of the breast tissue HER-2/neu status is one-time event, that cannot be used any further for follow up, the BM serve as a real-time indicator for HER-2/neu status during the management of the disease. However, frequent BM sampling is uncomfortably invasive procedure, hence a peripheral blood sample may be a more suitable option, especially if highly sensitive techniques as PCR were used. Because of its very important clinical significance, this suggestion needs to be confirmed with larger sample size.

In conclusion: BM micro-metastases can present in HER-2/neu positive breast cancer patients, in spite of being defined as clinically non-metastatic. We could not find any predicting factor for the presence of these BM micro-metastases, therefore, it has to be searched for in all HER-2/neu positive breast tissue patients. The detection of HER-2/neu Positive circulating cells carries a high possibility of BM metastases and may be used as an alternative to BM aspiration.

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REFERENCES

1. Paraskevas Paraskeva. The breast. In: Sir Alfred Cuschieri, Robert JC and Abdool Rahim Moossa eds. Arnold. New Yourk. 2002;3:68-88.

2. Armado E Giuliano. The breast. In: Lawrence W and Gerard M, eds. Current Surgical Diagnosis and Treatment. McGraw-Hill companies. New York. 2003;11:319-40.

3. Bjorn Naume, Elin Borgen, Gunnar Kvalheim, Rolf Kåresen, Hanne Qvist, Torill Sauer. Detection of Isolated Tumor Cells in Bone Marrow in Early-Stage Breast Carcinoma Patients: Comparison with Preoperative Clinical Parameters and Primary Tumor Characteristics. Clinical Cancer Research. 2001;7:4122–9.

4. Wang, H, Kåresen R, Hervik A, Thoresen S. Mammography screening in Norway: results from the first screening round in four counties and cost effectiveness of a modeled nationwide screening. Cancer Causes Controls. 2001;12:39–45.

5. JM Dixon. The breast. In: O James, Andrew W, John Forsythe and Graham Haddok, eds. Principles and Practice of Surgery. Churchill Livingstone. Edinburgh. 2002;4:409-22.

6. A Vincent-Salomon, F C Bidard, J Y Pierga. Bone marrow micro-metastasis in breast cancer: review of detection methods, prognostic impact and biological issues. Journal of Clinical Pathology. 2008;61:570-6.

7. Stephan Braun, Klaus Pantel. Clinical Significance of Occult Metastatic Cells in Bone Marrow of Breast Cancer Patients The Oncologist. 2001;6:125-32.

8. Stephan Braun, B. Semeni Cevatli, Cyamak Assemi, Wolfgang Janni, Christina R.M. Kentenich, Christian Schindlbeck, Dorothea Rjosk, and Florian Hepp. Comparative Analysis of Micrometastasis to the Bone Marrow and Lymph Nodes of Node-Negative Breast Cancer Patients Receiving No Adjuvant Therapy. Journal of Clinical Oncology. 2001;19:1468-75.

9. Pia Wulfing, Julia Borchard, Horst Buerger, Stefan Heidl, Kurt S. Za¨nker, Ludwig Kiesel,and Burkhard Brandt. HER2-Positive Circulating Tumor Cells Indicate Poor Clinical Outcome in Stage I to III Breast Cancer Patients. Clin Cancer Res. 2006;12:1715-20.

10. Stephan Braun, Gunter Schlimok, Isabella Heumos, Gerhard Schaller, Lutz Riethdorf, Gert Riethmuller, and Klaus Pantel. erbB2 Overexpression on Occult Metastatic Cells in Bone Marrow Predicts Poor Clinical Outcome of Stage I–III Breast Cancer Patients1. Cancer Research. 2001;61:1890–5.

11. Braun S, Pantel K, Muller P. Cytokeratin-positive bone marrow micro-metastases and survival of breast cancer patients with stage I-III disease. N Engl JMed. 2000;342:525-33.

12. lugo TG, Braum S, Cote RJ, Pantel K, Rusch V. Detection and measurement of occult disease for the prognosis of solid tumors. J clin Oncol. 2003;249:1552-5.

13. BC Pestalozzi, E Luporsi Gely, LM Jost, J Bergh. ESMO Minimum Clinical Recommendations for diagnosis, adjuvant treatment and follow-up of primary breast cancer. Annals of Oncology. 2005;16:7–9.

14. Jeffrey S Ross, Jonathan A Fletcher. The HER-2/neu Oncogene in Breast Cancer: Prognostic Factor, Predictive Factor, and Target for Therapy. The Oncologist. 1998;3:237-52.

15. Pinder, Francisco J. Esteva. A Patient With HER2-Positive Locally Advanced Breast Cancer Treated With Neoadjuvant Therapy. Case Studies in Breast Cancer. 2007;5:1-8.

16. LA Emens, RT Reilly, EM Jaffee Breast cancer vaccines: maximizing cancer treatment by tapping into host immunity. Endocrine-Related Cancer. 2005;12:1–17.

17. Richard G. Margolese. The palpable breast lump: information and recommendations to assist decision-making when a breast lump is detected. The Steering Committee on Clinical Practice Guidelines for the Care and Treatment of Breast Cancer. Can Med Assoc J. 1998;10:s3-s8.

18. Greene FL, Balch CM, Fleming ID. (eds): In AJCC (American Joint Committee on Cancer) Cancer Staging Handbook: TNM Classification of Malignant Tumors, sixth edition. New York: Springer-Verlag.2002.

19. Tom Bates, Kieran Horgan, Tim Archer, Julie Doughty, Robert Mansel, Zenon Rayter et al. Guidelines for the management of symptaomatic breast disease. EJSO. 2005;31:1–21.

20. Ronan A Cahill, Daniel Walsh, Rob J Landers, R Gordon Watson. Preoperative Profiling of Symptomatic Breast Cancer by Diagnostic Core Biopsy. Annals of Surgical Oncology. 2006;13:45-51.

21. Bernd Gerber, Annette Krause, Heiner Müller, Dagmar Richter, Toralf Reimer. Simultaneous Immunohistochemical Detection of Tumor Cells in Lymph Nodes and Bone Marrow Aspirates in Breast Cancer and Its Correlation with Other Prognostic Factors. Journal of Clinical Oncology. 2001;19:960-71.

22. A Vincent-Salomon, FC Bidard, JY Pierga. Bone marrow micrometastasis in breast cancer: review of detection methods, prognostic impact and biological issues. J. Clin. Pathol. 2008;61;570-6.

23. Shigeru Imoto, Takashi Kitoh and Takahiro Hasebe. Serum c-erbB-2 Levels in Monitoring of Operable Breast Cancer Patients. Japanese Journal of Clinical Oncology. 1999;29:336-9.