

Prognostic Significance of Occult Bone Marrow Micrometastases of Breast Cancer Detected by Quantitative Polymerase Chain Reaction for Cytokeratin 19 mRNA

Noriko Ikeda,¹ Yasuo Miyoshi,¹ Kazuyoshi Motomura,² Hideo Inaji,² Hiroki Koyama² and Shinzaburo Noguchi^{1,3}

¹Department of Surgical Oncology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871 and ²Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-0025

Amplification of cytokeratin 19 (CK19) transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) has been shown to be a highly sensitive assay for the detection of bone marrow micrometastases (BMM) of breast cancer, but recent studies have demonstrated the occurrence of false-positive results due to low-level, illegitimately transcribed CK19 in normal bone marrow tissue. One approach to solve this problem is to develop a quantitative CK19 RT-PCR assay and to introduce a cut-off value which can distinguish between illegitimate expression and cancer-specific expression levels. In the present paper, we describe a quantitative CK19 RT-PCR assay using a real-time automated PCR system. The number of CK19 transcripts was normalized to that of GAPDH transcripts as an internal control for quality and quantity of cDNA. The cut-off value for the ratio of CK19 to GAPDH transcripts was set at 10^{-4} since the ratio never exceeded this value in the control bone marrow samples ($n=12$). In total, 117 bone marrow aspirates from stage I–III patients with invasive breast cancers were subjected to CK19 RT-PCR assay and immunocytological examination. Forty (34.2%) were found to be BMM-positive by CK19 RT-PCR assay whereas only three (2.6%) were found to be BMM-positive by immunocytology. Multivariate analysis has shown that occult BMM detected by CK19 RT-PCR is a significant risk factor for relapse, being independent of axillary lymph node metastases.

Key words: Bone marrow — Micrometastases — Breast cancer — Quantitative RT-PCR

Bone marrow micrometastases (BMM) are reported to be found in 30–40% of breast cancer patients without clinical distant metastases by an immunocytological examination of the bone marrow aspirates using a variety of antibodies raised against epithelial cell-specific antigens.¹⁾ Most of these reports have concluded that BMM status serves as a significant prognostic indicator which is independent of the classical prognostic indicators such as lymph node status, histological grade, and tumor size.¹⁾ Therefore, today, BMM status is considered a useful parameter to define precisely the tumor stage, and it will be included in the TNM classification as a facultative prognostic factor designated M1_(i).²⁾

It has been shown that the detection rate of BMM increases in proportion to the number of bone marrow aspirates examined per patient.³⁾ Immunohistological examination of multiple bone marrow samples from six to eight sites including the sternum, bilateral anterior and posterior iliac crests, and sacrum usually gives a detection rate of BMM of around 30–40% in breast cancer patients without distant metastases but a single bone marrow sample shows BMM positivity only in 2–7%.³⁾ The requirement of multiple bone marrow sampling is a serious

problem which reduces the general acceptance of BMM examination as a routine procedure, since multiple samplings are not well accepted by patients, and, moreover, surgery is prolonged and a large number of immunohistological sections should be prepared in order to examine the whole sample.

One approach to solve this problem is the establishment of a sensitive method that can detect BMM using a single aspirate at a comparable detection rate to a conventional immunohistological method using multiple aspirates. Recently, several reports have indicated that amplification of cytokeratin 19 (CK19) mRNA, which is expressed in virtually all breast cancer cells, but not in hematopoietic cells in the bone marrow, by reverse transcriptase-polymerase chain reaction (RT-PCR) is a more sensitive method than immunocytology for the detection of BMM.^{4–6)} However, there has been controversy as to the specificity of CK19 RT-PCR assay. Some reports yielded no false-positive results in samples from healthy controls with CK19 RT-PCR^{4–6)} but other authors reported the occurrence of false-positive results at various frequencies.^{7–10)} A main source of false-positive results is thought to be amplification of low-level, illegitimately transcribed CK19 from hematopoietic cells.

Development of a quantitative CK19 RT-PCR assay might solve this problem by allowing the use of a cut-off

³ To whom correspondence should be addressed.

E-mail: noguchi@onsurg.med.osaka-u.ac.jp

value which is set using the control samples so as to differentiate illegitimate *CK19* expression and cancer-specific expression. Very recently, Slade *et al.* have developed a quantitative *CK19* RT-PCR assay which is based on estimation of the number of *CK19* transcripts by competitive PCR.¹¹ They have been able to demonstrate that the false-positive problem can be essentially solved by introducing an appropriate cut-off value and that the quantitative *CK19* RT-PCR assay which they developed is more sensitive than immunocytology in the detection of BMM. Their results are very interesting but the most important issue, i.e., the prognostic significance of the occult BMM detected by *CK19* RT-PCR, was not studied in their report. In addition, although they employed competitive PCR for quantification of *CK19* transcripts, a real-time automated PCR seems to be the method of choice because the latter method should be simpler and more suitable for a routine test than the former. Therefore, in the present paper, we have attempted to develop a quantitative *CK19* RT-PCR assay based on real-time automated PCR and, also, to study the prognostic significance of occult BMM detected by this new method.

MATERIALS AND METHODS

Bone marrow and peripheral blood sampling Bone marrow aspiration with a heparinized syringe was conducted immediately prior to surgery under general anesthesia for patients with stage I–III invasive breast cancer (*n*=117) or ductal carcinoma *in situ* (DCIS, *n*=12). The bone marrow aspirate (5 ml) was obtained from the sternum using a bone marrow aspiration needle. To eliminate the risk of skin contamination, an incision was made in the

skin before introducing the needle. The aspirate was semi-purified by Lymphoprep (Nycomed Pharma, AS., Oslo, Norway) gradient centrifugation, washed and resuspended in saline. Half of the sample was subjected to immunocytology and the other half was subjected to RNA extraction. Informed consent was obtained from every patient.

Peripheral blood was obtained through a venous catheter from the 15 healthy female volunteers; the first 1 ml was discarded because of possible contamination by epidermal cells, and then 10 ml was drawn using a new heparinized syringe. The peripheral blood samples were subjected to semipurification by Lymphoprep, and then to RNA extraction.

Immunocytology Bone marrow aspirate semipurified by Lymphoprep was smeared, fixed in ethanol/ether (4:1), and subjected to immunostaining of cytokeratins with the avidin-biotin-peroxidase method using anti-cytokeratin antibody (AE1/AE3) (Dako Japan Co., Ltd., Tokyo), which reacts to a broad spectrum of cytokeratins including cytokeratins 8, 18, and 19. Bone marrow smears were scored positive when cells stained strongly for cytokeratin and had the morphological characteristics of malignant cells.

RNA extraction Total RNA was extracted from the Lymphoprep-semipurified bone marrow aspirates and peripheral blood mononuclear cells as well as from MCF-7 breast cancer cells by the acid guanidium-phenol-chloroform technique using TRIzol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). The RNA extraction procedure was repeated two or three times until the interphase between the upper aqueous and lower phenol-chloroform phase became free of cell debris. The extracted RNA was resuspended in diethylpyrocarbonate-treated water and stored at -80°C until use.

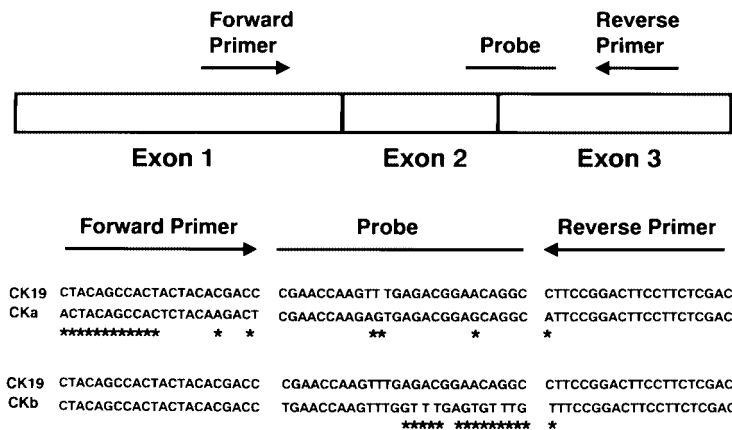


Fig. 1. Primers and internal probe for real-time PCR for *CK19* mRNA. Primers and internal probe were designed so as to maximize sequence difference between *CK19* and its pseudogenes (*CKa* and *CKb*). The internal probe was labeled with a reporter dye (FAM) at the 5'-end and a quencher dye (TAMRA) at the 3'-end. * Mismatches to *CK19*.

Real-time PCR for *CK19* mRNA We used real-time PCR (TaqMan PCR system, PE Applied Biosystems, Foster, CA) for quantification of *CK19* transcripts. The primers and internal probe for *CK19* are shown in Fig. 1. In order to prevent amplification from the processed pseudogenes (*CK19a* and *CK19b*),¹²⁾ primers and internal probes were designed to maximize the sequence difference between *CK19* and its pseudogenes. The internal probe for *CK19* was labeled with a reporter dye (FAM) at the 5'-end and a quencher dye (TAMRA) at the 3'-end. Real-time automated *CK19* PCR assay was conducted using TaqMan PCR master mix (PE Applied Biosystems) and an ABI 7700 Prism Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. In brief, first-strand cDNA was generated from 1 μ g of total cellular RNA in a 20 μ l reaction mixture containing 2.5 μ M oligo(dT)20, 5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl (pH 8.3), 1 mM d-NTP mixture, 1 mM dithiothreitol, 1 U/ μ l RNase inhibitor, and 0.25 U/ μ l of AMV reverse transcriptase (TaKaRa, Kyoto) for 30 min at 42°C. The reaction was terminated by heating at 95°C for 10 min.

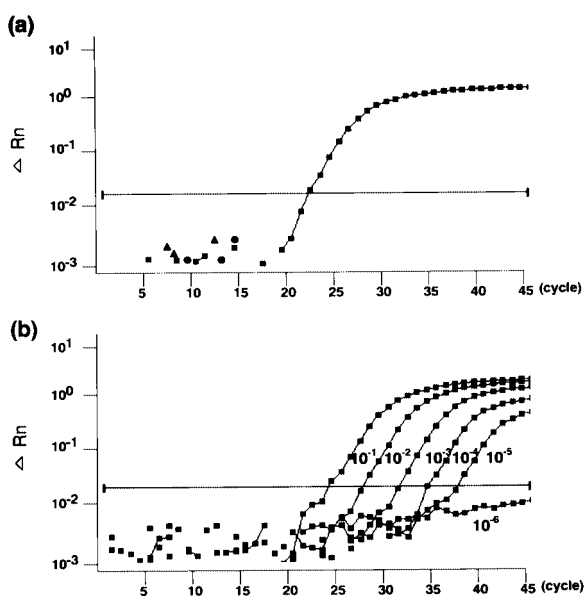


Fig. 2. Specificity and sensitivity of real-time PCR for *CK19* mRNA. (a) Specificity (upper panel): RNA from MCF-7 cells was subjected to real-time PCR with (■) or without (▲) a reverse-transcriptase pretreatment. DNA from MCF-7 cells was also subjected to real-time PCR (●). ΔRn (vertical axis) is the intensity of fluorescence of the reporter dye, representing the amount of the *CK19* amplicons. The horizontal axis represents the number of PCR cycles. (b) Sensitivity (lower panel): RNA from MCF-7 was diluted against RNA from peripheral blood mononuclear cells without *CK19* expression at various ratios from 10⁻¹ to 10⁻⁶, and subjected to reverse transcription and real-time PCR.

The PCR solution (50 μ l) was composed of 1 μ l of cDNA solution, 5 pmol of each of the forward and reverse primers, 10 pmol of internal probe and TaqMan Universal PCR Master Mix. PCR was carried out for 45 cycles (1 min at 94°C, and 0.5 min at 66°C) using the ABI 7700 Prism Sequence Detection System. PCR amplification of *GAPDH* mRNA was conducted according to the manufacturer's instructions using primers and the internal probe included in the *GAPDH* standard kit (PE Applied Biosystems).

Standard curves for *CK19* and *GAPDH* were generated using serial dilutions (10⁻¹ to 10⁻⁵) of RNA derived from breast cancer cell line (MCF-7 cells) in each assay, and the numbers of *CK19* and *GAPDH* transcripts were calculated according to these standard curves. The *CK19* PCR assay was performed in duplicate, and the mean value was used for calculation. *GAPDH* expression level was used as an internal standard for quality and quantity of cDNA and, thus, *CK19* expression level was expressed as a ratio of *CK19* to *GAPDH*.

Treatment of patients subjected to prognostic evaluation Prognostic significance of occult BMM, which could be detected by *CK19* RT-PCR assay but not by immunocytological examination, was evaluated in the 114 patients with invasive breast cancer and without immunocytologically detectable BMM. Median follow-up for these patients was 23 months with a range from 18 to 42 months. Sixty-five patients were treated with breast-conserving surgery followed by radiation therapy and 49 were treated with modified mastectomy. Ipsilateral breast recurrences after breast-conserving surgery were not counted as recurrences. Adjuvant chemo/endocrine therapy was mostly carried out as follows; patients with lymph node metastases received adjuvant chemotherapy (CMF (cyclophosphamide+methotrexate+5-FU)×6 cycles) and endocrine therapy (tamoxifen for 2 years) was added when estrogen receptor was positive. Patients without lymph node metastases received adjuvant chemotherapy if estrogen receptor was negative and tumor size exceeded 2 cm.

Statistics The relationship between occult BMM and other known risk factors was assessed by application of the χ^2 test. Disease-free survival rates were calculated according to the Kaplan-Meier method. Cox regression analysis (univariate and multivariate) was carried out to assess the impact of occult BMM on disease-free survival. These analyses on overall survival were not performed because the total number of deaths was too small.

RESULTS

Sensitivity and specificity of quantitative *CK19* RT-PCR assay When *CK19* RT-PCR assay was done on the MCF-7 cell-derived RNA samples pretreated with reverse transcriptase, a rapid, logarithmic increase of PCR prod-

ucts was observed, indicating a high expression level of *CK19* transcripts, whereas no PCR products were formed when *CK19* PCR assay was done on the same samples without reverse transcriptase pretreatment (Fig. 2a). In addition, *CK19* PCR assay of MCF-7 cell-derived genomic DNA resulted in no generation of *CK19* PCR products (Fig. 2a). These results demonstrate that false-positive results do not arise from amplification of the processed pseudogenes.

In order to estimate the sensitivity of *CK19* RT-PCR assay, serial dilution experiments were carried out. RNA derived from MCF-7 was diluted against RNA from peripheral blood mononuclear cells without *CK19* expression from 10^{-1} to 10^{-6} and subjected to *CK19* RT-PCR assay (Fig. 2b). *CK19* transcripts were detected up to 10^{-5} .

Fifteen samples of peripheral blood mononuclear cells from healthy controls were subjected to *CK19* RT-PCR. *CK19* expression (*CK19*:*GAPDH* ratio) was below the detection limit (10^{-5}) for 12 samples but three samples showed *CK19* expression ranging from 1.0×10^{-5} to 7.1×10^{-5} . Bone marrow samples were unavailable from healthy controls. Thus, we used bone marrow samples from patients with DCIS as controls, since DCIS is virtually a curable disease by surgery and unlikely to have BMM. *CK19* expression was undetectable for nine patients, but three patients showed *CK19* expression ranging from 1.0×10^{-5} to 5.2×10^{-5} . Since no samples from these controls showed *CK19* expression above or equal to 10^{-4} , this value was used as a cut-off value for *CK19* expression in the following assays (Fig. 3).

Incidence of BMM In total, 117 patients with stage I ($n=43$), stage II ($n=68$), or stage III ($n=6$) invasive breast cancers (113 invasive ductal cancers and four invasive lobular cancers) were analyzed by both *CK19* RT-PCR assay and immunocytological examination. Of these patients, 40 patients (34.2%) were found to be positive by the *CK19* RT-PCR assay, whereas only three patients (2.6%) were found to be positive by immunocytology (Table I, Fig. 3). *CK19* RT-PCR positivity was higher in the patients with lymph node metastasis as compared with those without it. All three patients with positive immunocytological results were also positive by *CK19* RT-PCR.

Clinicopathological characteristics of breast cancers with occult BMM Of the 117 patients with primary breast cancers, the three patients with positive results in both *CK19* RT-PCR assay and immunocytology were excluded, and the remaining 114 patients were analyzed herein in order to elucidate the clinicopathological characteristics of the patients with occult BMM which could be detected by *CK19* RT-PCR assay but not by immunocytology (Table II). Positivity of axillary lymph node metastases ($P < 0.05$) and lymphatic vessel invasion ($P < 0.05$) were significantly higher in the *CK19* RT-PCR assay-positive patients than the negative patients. There was no

significant difference in other factors between the two groups.

Prognostic significance of occult BMM The prognostic significance of occult BMM was evaluated in the 114 immunocytologically BMM-negative patients. Patients with *CK19* RT-PCR positive results showed a significantly ($P < 0.05$) poorer disease-free survival than those with negative results (Fig. 4). Univariate Cox regression analysis revealed that the positive *CK19* RT-PCR assay result was a significant risk factor for relapse, and other risk

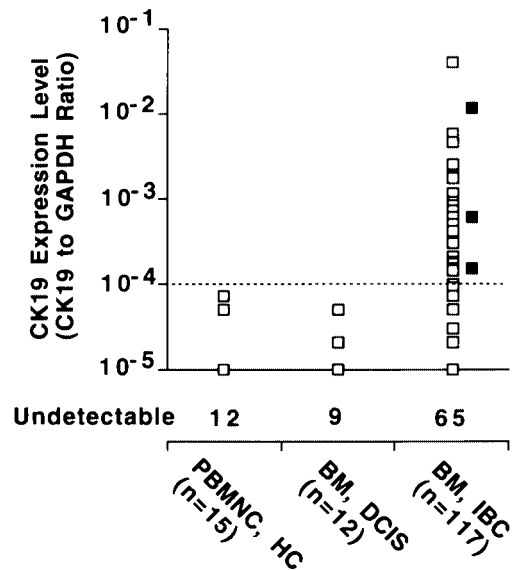


Fig. 3. Quantitative PCR assay for *CK19* mRNA of peripheral blood and bone marrow samples. RNA samples from peripheral blood and bone marrow were subjected to reverse transcription and real-time PCR for *CK19* mRNA. *CK19* expression levels were expressed as ratios of *CK19* to *GAPDH* (internal control). *CK19* expression was undetectable in 12 of 15 peripheral blood mononuclear cells from healthy controls (PBMNC, HC), in 9 of 12 bone marrow samples from patients with DCIS (BM, DCIS), and in 65 of 117 bone marrow samples from patients with invasive breast cancer (BM, IBC). ■ represents three patients with immunocytologically detectable bone marrow micrometastases.

Table I. Incidence of Bone Marrow Micrometastases of Breast Cancer Detected by *CK19* RT-PCR and Immunocytology

	No. of patients	<i>CK19</i> RT-PCR	Immunocytology
Lymph node status			
Negative	74	21 (28.4%)	1 (1.4%)
Positive	43	19 (44.2%)	2 (4.9%)
Total	117	40 (34.2%)	3 (2.6%)

factors such as large tumor size, lymph node metastases, lymphatic vessel invasion, high histological grade, and negative estrogen receptor status were also found to be significant (Table III). Then, multivariate Cox regression analysis (forward stepwise method) was carried out to assess the independency of each risk factor from others. This showed that a positive *CK19* RT-PCR result was a significant risk factor independent of tumor size, lymph node status, and estrogen receptor status.

DISCUSSION

Low specificity of conventional *CK19* RT-PCR seems to be attributable partially to amplification of the pseudogenes from contaminating genomic DNA and mainly to amplification of the *CK19* illegitimately transcribed from normal hematopoietic cells. Since *CK19* has two highly homologous pseudogenes (*CKa* and *CKb*), it is very important to maximize the sequence difference when primers for RT-PCR are designed. Our method, TaqMan PCR, has an advantage as compared with the conventional

Table II. Histologic Characteristics of Breast Cancers with Bone Marrow Micrometastases Detected by *CK19* RT-PCR^{a)}

		<i>CK19</i> RT-PCR	
		Positive (n=37)	Negative (n=77)
Age (years)	≤50	20 (54%)	39 (51%)
	>50	17 (46%)	38 (49%)
Tumor size (mm)	≤20	16 (43%)	31 (40%)
	20< ≤50	20 (54%)	42 (55%)
Lymph node metastases	Negative	18 (49%)	55 (71%) ^{b)}
	Positive	19 (51%)	22 (39%)
Histological grade	Grade I	12 (32%)	22 (29%)
	Grade II	9 (24%)	20 (26%)
	Grade III	16 (44%)	35 (45%)
Lymphatic vessel invasion	Negative	25 (68%)	65 (84%) ^{b)}
	Positive	12 (32%)	12 (16%)
Estrogen receptor	Negative	23 (62%)	37 (48%)
	Positive	14 (38%)	40 (52%)
Location of tumor	Medial	12 (32%)	37 (48%)
	Lateral	25 (68%)	40 (52%)

a) Three patients with immunocytologically detectable bone marrow micrometastases were excluded.

b) *P*<0.05.

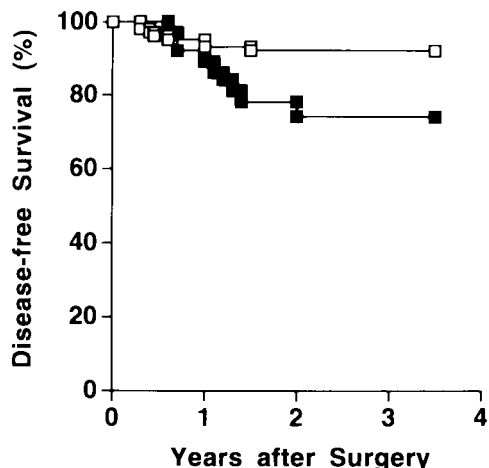


Fig. 4. Disease-free survival of breast cancer patients in relation to quantitative *CK19* RT-PCR assay results. Disease-free survival rates were calculated for the 114 patients with invasive breast cancer and without immunocytologically detectable bone marrow micrometastases. Patients with *CK19* RT-PCR positive results (■) showed a significantly (*P*<0.05) poorer disease-free survival than those with negative results (□).

Table III. Prognostic Significance of *CK19* RT-PCR Assay Results Studied by Univariate and Multivariate Analysis

Risk/reference factors	Univariate		Multivariate ^{a)}		
	RR	<i>P</i> value	RR	<i>P</i> value	
Age (years)	≤50/>50	1.4	0.497		
Tumor size (cm)	>5/≤5	6.7	0.003	9.8	0.006
Lymph node metastases	+ve ^{c)/-ve^{d)}}	5.8	0.002	8.9	0.005
Lymphatic vessel invasion	+ve/-ve	3.9	0.016		NS ^{b)}
Histological grade	III/I+II	5.2	0.010		NS ^{b)}
Estrogen receptor	-ve/+ve	8.2	0.005	9.0	0.007
<i>CK19</i> RT-PCR assay	+ve/-ve	3.2	0.028	7.1	0.004

a) Six significant risk factors revealed by univariate analysis were further analyzed by the multivariate method (forward stepwise method).

b) Not significant.

c) Positive.

d) Negative.

PCR that inclusion of an internal probe besides the primers can enhance the specificity. The fact that amplification of *CK19* products was neither observed in MCF-7 cell-derived RNA without a reverse-transcriptase pretreatment or in MCF-7 cell-derived genomic DNA indicates that the *CK19* RT-PCR assay developed in the present study does not amplify the pseudogenes.

In order to eliminate false positives resulting from amplification of the illegitimate *CK19* expression from hematopoietic cells, we attempted to set a cut-off value which could distinguish the cancer-specific expression from illegitimate expression. Low-level expression of *CK19* was observed in a few control samples from peripheral blood and bone marrow but it never exceeded 10^{-4} (*CK19*-to-*GAPDH* ratio). Thus, we adopted this value as a cut-off value. Slade *et al.* reported that the cut-off values in their competitive PCR assay were 1/1000 for blood samples and 1/1600 for bone marrow.¹¹ This difference in cut-off values seem to be attributable to the different methods used for quantification of *CK19* transcripts.

Of the 117 breast cancer patients, only three (2.6%) were revealed to have BMM by the immunocytological examination of a single bone marrow aspirate in the present study. This incidence of BMM is apparently lower than those (30–40%) reported in multiple bone marrow aspirates from six to eight different sites.¹ Such a low incidence of BMM in a single bone marrow aspirate is consistent with previous reports,³ indicating that immunocytological examination of a single marrow aspirate is clinically useless. On the other hand, quantitative *CK19* RT-PCR assay was more sensitive than immunocytology. The incidence of BMM detected by quantitative *CK19* RT-PCR assay was 34.2% (40/117). Slade *et al.* have also reported that quantitative *CK19* RT-PCR (competitive PCR) is more sensitive than immunocytology in the detection of BMM.¹¹

Clinical significance of immunocytologically detected BMM has been reported frequently¹ but that of occult BMM, which can be detected by *CK19* RT-PCR but not by immunocytology, has yet to be established. In the present study, we have been able to demonstrate that occult BMM are significantly associated with lymph node metastases and lymphatic vessel invasion, and that the prognosis of the patients with occult BMM is significantly poorer than that of patients without them. Furthermore, it has been shown by multivariate analysis that occult BMM can serve as a significant risk factor for relapse, being independent of lymph node metastases, tumor size, and estrogen receptor status. Diel *et al.*¹³ and Harbeck *et al.*¹⁴ have also reported that BMM are an independent prognostic factor by multivariate analysis, using a median follow-up of about 3 years but, very recently, Mansi *et al.* have reported that BMM are not an independent prognostic factor in a study with a longer median follow-up of 12.5

years.¹⁵ Therefore, the significance of occult BMM as an independent prognostic factor demonstrated in the present study still remains to be established by further study with a longer follow-up.

Of the 37 patients with occult BMM, nine developed recurrences, i.e., two in the bone (including one sternal metastases), two in the liver, two in the lung, two in the regional lymph nodes, and one in the local skin. Therefore, the presence of occult BMM is not a predictor of bone recurrences. Rather, it is indicative of a high likelihood of recurrence in any organ, as is the case for BMM detected by immunocytology.¹

Since bone marrow samples were not available from healthy controls, we used bone marrow samples from the patients with DCIS as controls. We confirmed the diagnosis of DCIS by histological examination of multiple sections from each lesion, and we consider that DCIS included in the present study is pure DCIS which is curable by surgery alone and virtually devoid of risk for developing distant metastases. Therefore, we think that usage of bone marrow samples from DCIS patients as controls is acceptable. However, the possibility can not be ruled out completely that microinvasion was overlooked in DCIS patients and exceptionally, the patients had BMM. Thus, the cut-off value which was decided in the present study should be re-evaluated in the future based on a larger number of bone marrow samples from healthy controls.

Among the *CK19* RT-PCR assay-positive patients, the *CK19* expression levels varied from 1.0×10^{-4} to 3.9×10^{-2} . It is speculated that the higher *CK19* expression levels might reflect a higher tumor burden in the bone marrow, and that they might be associated with an even higher relapse rate among the *CK19* RT-PCR assay-positive patients. However, such association was not observed in the present study (data not shown). *CK19* RT-PCR assay can detect the presence of cancer cells, but it can not distinguish whether or not these cancer cells are capable of growing to form clinically detectable metastatic foci. Not only quantity but also quality of cancer cells is important for the establishment of metastatic foci. Heterogeneity in quality of cancer cells from patient to patient may explain why the higher *CK19* RT-PCR assay values were not associated with a higher relapse rate.

Finally, in the present study, we have established a quantitative *CK19* RT-PCR assay using a real-time automated PCR and have been able to demonstrate that this assay is more sensitive than immunocytology in the detection of BMM of breast cancers, and that occult BMM can serve as a significant and independent risk factor for relapse. It is suggested that the quantitative *CK19* RT-PCR assay of a single bone marrow aspirate is clinically as useful as immunocytological examination of multiple bone marrow aspirates for the prediction of patient outcome. A

conclusion can not be reached until the results of studies including a larger number of patients with a longer follow-up period are obtained, but our present results seem to warrant such future studies since, unlike immunocytological examination of multiple bone marrow aspirates, quantitative *CK19* RT-PCR assay of a single bone marrow aspirate is much easier and, thus, practically acceptable.

REFERENCES

- 1) Funke, I. and Schraut, W. Meta-analysis of studies on bone marrow micrometastases: an independent prognostic impact remains to be substantiated. *J. Clin. Oncol.*, **16**, 557–566 (1998).
- 2) Hermanek, P. pTNM and residual tumor classifications: problems of assessment and prognostic significance. *World J. Surg.*, **19**, 184–190 (1995).
- 3) Giai, M., Natoli, C., Sismondi, P., Biglia, N., Lesti, G., Mulieri, M. and Lacobelli, S. Bone marrow micrometastases detected by a monoclonal antibody in patients with breast cancer. *Anticancer Res.*, **10**, 199–222 (1990).
- 4) Datta, Y. H., Adams, P. T., Drobyski, W. R., Ethier, S. P., Terry, V. H. and Roth, M. S. Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J. Clin. Oncol.*, **12**, 475–482 (1994).
- 5) Schoenfeld, A., Kruger, K. H., Gomm, J., Sinnett, H. D., Gazet, J. C., Sachs, N., Blender, H. G., Luqmani, Y. and Coombes, R. C. The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19. *Eur. J. Cancer*, **33**, 854–861 (1997).
- 6) Fields, K. K., Moscinski, L. C., Janssen, W. E., Perkins, J. B., Trudeau, W. L. and Eifenbein, G. J. Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. *J. Clin. Oncol.*, **14**, 1868–1876 (1996).
- 7) Aihara, T., Noguchi, S., Ishikawa, O., Furukawa, H., Hitatsuka, M., Ohigashi, H., Nakamori, S., Monden, M. and Imaoka, S. Detection of pancreatic and gastric cancer cells in peripheral and portal blood by amplification of keratin 19 mRNA with reverse transcriptase-polymerase chain reaction. *Int. J. Cancer*, **72**, 408–411 (1997).
- 8) Bostick, P. J., Chatterjee, S., Chi, D. D., Huynh, K. T., Giuliano, A. E., Cote, R. and Hoon, D. S. B. Limitations of specific reverse-transcriptase polymerase chain reaction

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

(Received May 8, 2000/Accepted June 22, 2000)

- markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. *J. Clin. Oncol.*, **16**, 2632–2640 (1998).
- 9) Novaes, M., del Giglio, A., Garicochea, B. and Bendit, I. Reverse transcriptase-polymerase chain reaction analysis of cytokeratin 19 expression in the peripheral blood mononuclear cells of normal female blood donors. *Mol. Pathol.*, **50**, 209–211 (1997).
- 10) Lopez-Guerrero, J. A., Bolufer-Gilabert, P., Sanz-Alonso, M., Barragan-Gonzalez, E., Palau-Perez, J., De la Rubia-Comos, J., Sempere-Telens, A. and Bonanad-Boix, S. Minimal illegitimate levels of cytokeratin K19 expression in mononucleated blood cells detected by a reverse transcription PCR method (RT-PCR). *Clin. Chim. Acta*, **263**, 105–116 (1997).
- 11) Slade, M. J., Smith, B. M., Sinnett, H. D., Cross, N. C. P. and Coombes, R. C. Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. *J. Clin. Oncol.*, **17**, 870–879 (1999).
- 12) Ruud, P., Fodstad, O. and Hovig, E. Identification of a novel cytokeratin 19 pseudogene that may interfere with reverse transcriptase-polymerase chain reaction assays used to detect micrometastatic tumor cells. *Int. J. Cancer*, **80**, 119–125 (1999).
- 13) Diel, I., Kaufmann, M., Costa, S. D., Holle, R., von Minckwitz, G., Solomayer, E. F., Kaul, S. and Bastert, G. Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. *J. Natl. Cancer Inst.*, **88**, 1652–1658 (1996).
- 14) Harbeck, N., Untch, M., Pache, L. and Eiermann, W. Tumour cell detection in the bone marrow of breast cancer patients at primary therapy: results of a 3-year median follow-up. *Br. J. Cancer*, **69**, 566–571 (1994).
- 15) Mansi, J. L., Gogas, H., Bliss, J. M., Gazet, J. C., Berger, U. and Coombes, R. C. Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study. *Lancet*, **354**, 197–202 (1999).