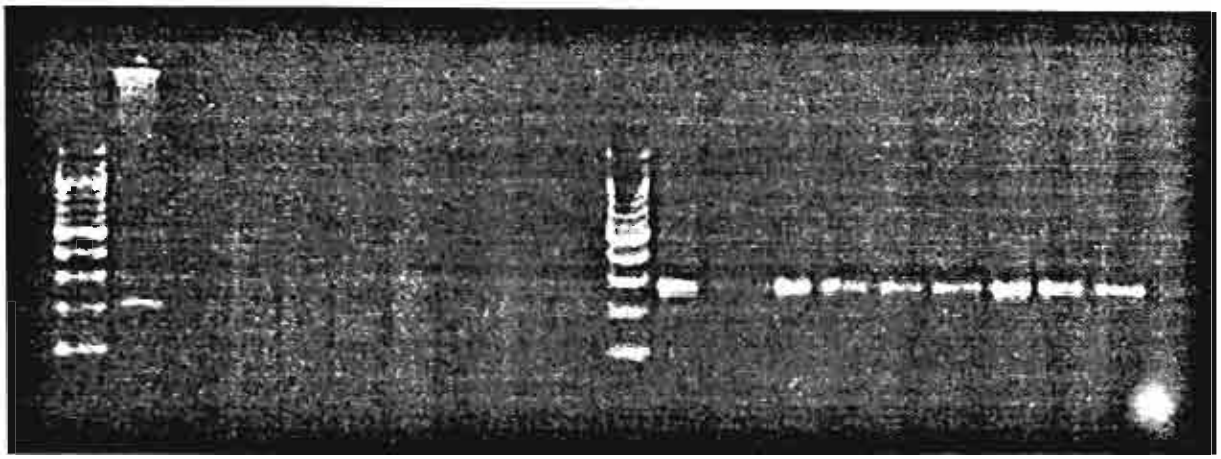

Figure 7. Ethidium bromide staining of agarose gel showing nested RT-PCR analysis of β_2–microtubulin and mammaglobin mRNA expression in peripheral blood samples of female patients with malignancies other than breast cancer.

lane M : molecular weight marker (100 bp DNA ladder)

lane P : positive control

lane N : water (blank)

Table 2. Detection of circulating mammary carcinoma cells by *hMAM* nested RT-PCR

Tumour type	Number	*hMAM*-positive	*hMAM*-negative	*P*-value
Breast cancer	200	64	136	
Other malignancies	30	0	30	<0.001
Healthy control	30	0	30	<0.001

3. The correlation between *hMAM* expression in circulating mammary carcinoma cells and clinicopathological prognostic factors of breast cancer

To investigate whether the presence of *hMAM*-expressing breast cancer cells in blood preoperatively was correlated with clinical prognosis, we performed a statistical analysis including almost all of the related prognostic factors of breast cancer. Although *hMAM* mRNA was found in breast cancer patients rather than in healthy controls and those with other cancers, the presence of these cells was not associated with any clinicopathological prognostic factors (Table 3). No significant difference in age distribution, proportion of menopausal patients, or tumour characteristics was detected. To avoid a potential false-positive result due to the age difference, these patients were divided into two age groups: ≤ 50 years of age, and ≥ 50. Again, we found no difference in *hMAM* transcripts among the groups.

The difference in *hMAM* transcripts detected between stage I and II, and III and IV was also not significant (24.7% vs. 8.1%, $P = 0.5890$). The level of positive results for the moderately differentiated group seems higher than for the well-differentiated and poorly differentiated groups, but the number of patients with well differentiation was too small to make an adequate comparison. Similarly, comparison between poor differentiation and non-poor differentiation (well and moderately differentiated) also showed no statistical significance (9.9% vs. 22.5%, $P = 0.5220$). The three prognostic factors representing tumour extension and metastasis (i.e., lymphatic invasion, vascular invasion, and lymph node metastasis) also were not related to *hMAM* positive transcripts.

Table 3. Characteristics of 200 breast cancer patients and tumours according to the results of nested RT-PCR

Factors	nested RT-PCR		*P*
	hMAM (+) (n=64)	*hMAM* (-) (n=136)	
Patient age (yrs)			
Mean ± SD	49.56±10.2	51.54±11.1	0.2300[a]
Median			
≤ 50 yrs	39	66	
> 50 yrs	70	25	0.1370[b]
Menopausal status			
Premenopause	36	62	
Postmenopause	28	74	0.2090[b]
Tumour size (cm)			
≤ 2	30	59	
> 2 and ≤ 5	25	63	
> 5	9	14	0.9784[b]
Stage			
I	46	88	
II,III and IV	15	37	0.9753[b]
Regional lymph nodes			
Positive	30	77	
Negative	34	59	0.2560[b]
Histological type			
Noninvasive ductal carcinoma	3	11	
Invasive ductal carcinoma	61	125	0.5550[b]
Oestrogen receptor status			
Positive	27	50	
Negative	19	32	0.8004[b]
Unknown	18	54	
Progesterone receptor status			
Positive	22	30	
Negative	21	49	0.1590[b]
Unknown	21	57	
Histological grade			
Well	6	8	
Moderately	35	70	
Poorly	18	45	0.2988[b]
Lymphatic invasion			
Positive	4	11	
Negative	60	125	0.7780[b]
Vascular invasion			
Positive	5	10	
Negative	59	125	1.0000[b]

[a] Student's *t* test
[b] χ^2 test

Discussion:

1. The establishment of nested RT-PCR technique and the identification of human mammaglobin *(hMAM)* mRNA in circulating mammary carcinoma cells

1.1 RNA extraction

We successfully extracted RNA from peripheral blood of healthy volunteers, patients with various malignancies others than breast cancer and breast cancer patients. RNA is not very stable and can be easily destroyed by endogenous RNases. Therefore, certain conditions are necessary for RNA extraction. For example, the process should be extremely quick and the temperature should be chosen carefully. Besides, to avoid the contamination of probes with RNases, the solution should be prepared with RNase-free water. The most frequently used method is acid guanidine-phenol extraction. This method destroys cells, dissolves their components and at the same time denatures endogenous RNases. Extracted RNA is precipitated with alcohol, then dissolved in water or buffer and is stored at a low temperature.

1.2 The establishment of nested RT-PCR

Nested RT-PCR is a modified version of PCR that provides identification of disseminated carcinoma cells by targeting tumour specific mRNA in peripheral blood. Whether a nested RT-PCR is useful depends not only on the marker used but also on the assay conditions for that marker. As a consequence, many parameters were optimised in nested RT-PCR reactions. The sensitivity and specificity of this method should be a function of several parameters, including template concentrations, the concentration of salts, pH and annealing temperature. We concluded that reproducibility of nested RT-PCR is affected by the template RNA concentration. In addition, buffer components, such as magnesium, have been shown to influence nested RT-PCR reactions as well as in those standard PCR-based techniques.

The nested RT-PCR technique has been successfully established. It is a rapid and powerful technique for detecting a small number of tumour cells in a background of many normal cells, and has been widely used to detect micrometastatic cells. PCR amplification for tumour-specific DNA sequence abnormalities has been generally used in haematological malignancies and colorectal cancer. The main disadvantage of

PCR assay for DNA sequence abnormalities in tumour is that it may detect not only the DNA of viable tumour cells, but also the DNA fragments of non-viable carcinoma cells. For breast cancer, the detection of circulating cancer cells has been focused on tissue-specific mRNAs, since the normal blood cells do not express these genes. A positive nested RT-PCR result indicates the presence of viable carcinoma cells due to the instability of mRNA.

2. Detection of the circulating mammary carcinoma cells in normal, breast cancer patients and patients with various malignancies other than breast cancer

The ability to detect malignant spread at its early stage is desirable, because this may have important prognostic and therapeutic implications. At present, tumour metastasis is diagnosed by clinical manifestations and imaging studies concurrently with serum marker assays. These techniques are only beneficial in later stages of tumour spread because of the need for a critical minimal tumour volume. Sensitive immunocytologic tests for the detection of single tumour cells in the peripheral blood of cancer patients were exploited. Nevertheless, the antibodies used had some degree of false positivity, and this, as a result, limited their prognostic value.

Nested RT-PCR allows the specific detection of tumour cells at the mRNA level in secondary sites like peripheral blood or bone marrow with an analytical sensitivity of one tumour cell measurable in background of 10^6 normal cells without the need of tumour cells enrichment or purification prior to analysis. In contrast to other tumours, specific marker genes for the detection of disseminated cancer cells in blood of breast cancer patients are not yet available. Most tumour-specific gene (e.g. CEA) or epithelium-specific genes (e.g. Cytokeratin 19) exhibited limited diagnostic value because of either ectopic expression or limited sensitivity and specificity. Thus, tissue and/or tumour specific marker genes are needed for the detection of metastases in the peripheral blood of breast cancer patients.

Mammaglobin, a glycoprotein distantly related to the uteroglobin gene family, seemed to become a promising candidate for a breast cancer specific marker with regard to tumour specificity and sensitivity allowing also the detection of tumour cell dissemination in axillary lymph nodes and blood. As the characterisation of tumour cell dissemination is a main focus of our study, we established a nested RT-PCR test for the detection of *hMAM* expression in peripheral blood of Thai breast cancer

patients. The test established proved to be a sensitive and robust method to detect mRNA expression.

With respect to specificity, *hMAM* expression was only detectable in blood of breast cancer patients, but not in the control group of apparently healthy females. Furthermore, no *hMAM* mRNA expression was detected in all peripheral blood samples from patients with malignancies other than breast cancer. This indicated high specificity of *hMAM* as a marker gene for cells derived from mammary glands. Because none of our healthy controls tested positive, the high specificity of our assay was compatible with other reports using RT-PCR to detect the mRNA of epithelial markers. However, there are enough studies that report high false-positive rates in healthy controls to question this method's use for clinical diagnosis. The sensitivity of RT-PCR commonly varies and may be caused by a lack of standardisation, including criteria for patient selection, sample collection, RNA extraction, PCR conditions, and primers designed. The high specificity of our assay seems to be related to our careful protocol. To confirm pure RNA preparation, the samples were checked periodically for genomic DNA contamination using RNA as the template for PCR assay.

In peripheral blood of 200 breast cancer patients, significant expression of *hMAM* mRNA was detectable in 64/200 (32%) of samples. Zach *et al* also reported that 25% of breast cancer patients exhibited *hMAM* expression in peripheral blood It remains to be evaluated in prospective studies whether tumour cells in peripheral blood detected by *hMAM* expression are of any prognostic value, especially for patients at diagnosis without advanced disease and for patients with no evidence of disease.

3. The correlation between *hMAM* expression in circulating mammary carcinoma cells and clinicopathological prognostic factors of breast cancer

Although node-negative breast cancer patients have a favourable prognosis, recurrent disease occurs in approximately 20% of patients within 10 years after surgery. Therefore, considerable efforts have been made to find markers conclusively associated with risk of relapse. As clinical outcomes in breast cancer often require a prolonged follow-up, which is still in progress for the current study, *hMAM* expression was evaluated against known prognostic clinicopathological factors for correlation.

RNA-based assays by epithelial markers of tumour cells in the blood have shown preliminary clinical significance, given in brief as in Table 4. According to a CEA mRNA assay, the level positive breast cancer cells in the blood were associated with increased stage, suggesting a concept of molecular pathology. However, there are only a few similar studies on breast cancer patients. It appears that analysing blood for tumour cells is a promising prognostic tool.

In our study, there was no statistical significance of the potential correlations between *hMAM* mRNA in circulating cell and prognostic clinicopathological factors. *hMAM* mRNA was not related to tumour size, stage, histological type, differentiation status oestrogen receptor and progesterone receptor status, lymph node metastases, lymphatic invasion, vascular invasion, menopausal status or patient age. Paradis *et al.* and Yuan *et al.* similarly reported poor correlations between tumour cells in blood, as determined by RT-PCR of biomarker mRNA, and prognosis in prostate cancer and cervical cancer patients respectively. Therefore, the utility of this assay has not yet been confirmed.

There are limited data on the clinical application of nested RT-PCR assay in the detection of circulating breast cancer cells. Most studies trying to correlate prognostic value with the detection/ presence of circulating breast cancer cells are prospective and have very limited data on follow up. It is expected that an extended follow up would provide more decisive information. For example, detection of maspin in the circulation using RT-PCR showed an increased level of circulating breast cancer cells after chemotherapy. Therefore, nested RT-PCR assay may provide an additional tool for the prediction of metastasis.

Table 4. Clinical significance of RNA-based assay of tumour cells in blood by epithelial markers detected through RT-PCR

Investigators	Cancer site	Epithelial markers	Clinical significance
Mori *et al.*	Breast	CEA	Increased stages
Yeh *et al.*	Stomach	CK19	Poor survival; nonreponsiveness to chemotherapy
Stenman	Cervix	SCC-Ag	Poor prognosis
Katz *et al.*	Prostate	PSA	Reflected capsular penetration and positive surgical margin
Peck *et al.*	Lung	CK19	Reflected tumour burden and nonresponsiveness to treatment
Pao *et al.*	Cervix	HPV 16[a]	Existed in 86.7% of advance stages

Note. SCC-Ag (squamous cell carcinoma antigen), PSA (prostate-specific antigen)

[a] HPV16-E6 transforming gene

Conclusion:

Mammaglobin RT-PCR exhibited a specific method to detect circulating breast cancer cells. While it detects one breast cancer cell in 10 to 10 million white blood cells, none of the samples from peripheral blood of 30 healthy individuals were positive, whereas 64 (32%) of 200 samples from breast cancer patients were positive for human mammaglobin (*hMAM*) mRNA.

The clinical importance of the circulating cancer cells of breast cancer has become increasingly recognised. The past decade has been a dramatic increase in the output of studies in this area. The clinical, diagnostic and therapeutic value of detecting circulating cancer cells has begun to unfold. Given the limits of the current reported results, improvements can be made to enhance the study in the molecular detection of circulating cancer cells in breast cancer.

It seems sensible to study these patients continually to clarify the long-term effect of the positive assays on prognosis, possibly for 5-10 years. Adding patients with advanced breast cancers would be more informative for this study, and observing these patients during chemotherapy to see the cell positivity after each course of chemotherapy in comparison to prechemotherapy positivity would add significant understanding as to the possible clinical-pathologic importance of cell positivity.

In conclusion, the detection of circulating cancer cells in breast cancer patients may indicate an early phase of haematogenous spreading that possibly will develop further into metastasis or recurrence. Although the long-term effect of this phenomenon has not been clarified yet, especially regarding survival, this assay provides a model for monitoring early dissemination of cancer cells and finding factors to overcome this problem, which may ultimately allow us to prevent breast cancer from metastasising or recurring.

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