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医療費削減と患者負担軽減をめざした癌の新しい分子遺伝学的診断法の開発

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目　　次

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統合研究報告書

医療費削減と患者負担軽減をめざした癌の新しい分子遺伝学的診断法の開発

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研究要旨　個々の癌患者の再発予測が可能になれば、抗癌剤治療が必要な患者のみを選択できる可能性があり、不要な患者への投与を回避できることにより、医療費の軽減が実現する。われわれは再発予測の指標として、subclinical levelでの診断を目指し末梢血中および骨髄中の微量癌の存在に注目し、平成17年度までに厚生労働省科学研究費補助金（がん克服戦略研究事業）により、最終目標2000例の乳癌・消化器癌における末梢血・骨髄の微量癌検出を開始した。平成16年度末までに合計1714症例を採取していたが、平成17年度の1年間で合計2363例（乳癌1214例、食道癌17例 胃癌1035例 大腸癌52例 その他16例 健常コントロール29例）に到達しており、平成17年度には予定の2000例を越える症例のサンプル集積を終えた。　平成１７年度には、RT-PCR法を用いて臨床病理学的因子等の患者情報が揃った乳癌855例と胃癌910例について末梢血中および骨髄中の遊離がん細胞を検索した。その結果乳癌では、術後1年経過した633症例に限ると末梢血液中のCK陽性200例は陰性433例に比し、統計学的有意差を持ち、overall survaivalが低い事が明らかになった。従って、乳癌では、末梢血液に存在する遊離癌細胞自体が予後・転移・再発能を決定しうる、真の癌細胞であると考えられた。

一方、胃癌症例では、臨床病期におけるCEAまたはCK7発現頻度は末梢血液、骨髄ともに差がなく、真の転移を規定する遊離癌細胞を解析する必要があると考えられた。

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【A】研究目的

1) 乳癌症例において遊離癌細胞(ITC)検出の至適マーカーを決定。再発・予後との関係を明らかにする。
2) ITCの中に転移形成能を有するものと有さないものがあると考えられるため、“真の転移形成能”を有するITCの検出マーカーを同定する。

【B】研究方法

1) ITCの検出：至適マーカーの検討：株化癌細胞を用いて、ITC検出のため最適(高い特異性と感受性)遺伝子を同定。2000年6月から3施設で登録した855例の乳癌患者及び胃癌910例の骨髄および末梢血液を用いて、hybridization法でITCを検出。
2) 真の転移能を有するITCの同定：真の転移能を有すると考えられる『癌幹細胞様』細胞(SP細胞)を採取し、特異的に発現する遺伝子を同定する。骨髄液より癌細胞の単離培養を施行し、増殖能を調べてマーカーを同定する。
3) 宿主(host)を含めた転移マーカーの同定：非再発例に比し再発陽性症例の骨髄液中、過剰発現している遺伝子を明らかにする。

【C】研究結果

1)臨床症例を用いた解析
(1)平成16年度に報告した如く、乳癌細胞6株、胃癌細胞11株、大腸癌細胞10株、非上皮系細胞8株を対象にして、CEA, CK7, CK18, CK19, CK20, Mam, Muc1遺伝子を標的遺伝子候補にしたRT-PCRを行い、癌細胞株で陽性率が高く、正常株で陰性である最適なマーカーを決定した。その結果、CK7が大腸癌、胃癌、乳癌いずれにおいても感受性、特異性ともに高く最

適と考えられた。

表１至適マーカーの同定

細胞株（数）	CEA	CK7	CK19	CK20	MMG
乳癌（4）	75	100	100	25	75
胃癌（12）	25	100	91.7	25	41.7
大腸癌（11）	63.6	100	90.9	72.7	18.3

尚、非上皮系株化細胞８種類の上限値をcut-off値として、その発現を調べた。

(2) 乳癌症例において、 CK7を標的遺伝子にした解析の結果、術後１年経過した633症例に限り、末梢血液におけるCK7陽性200症例は陰性433症例に比較して、統計学的有意差を持ち予後が悪いことが明らかになった。臨床病理学的因子に関しては、特にCK7発現との相関を認めなかった。しかし、今回の解析においては、骨髄中におけるCK7の発現は特に予後・再発等との相関を認めなかった。

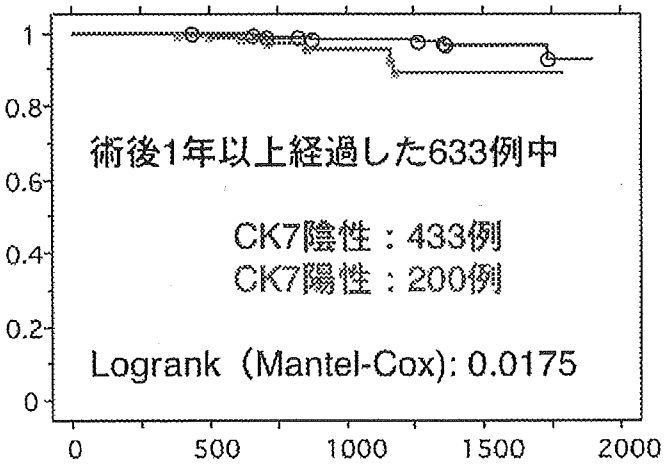

図1 乳癌末梢血液中CK7発現と予後との関係

(3)胃癌はCEA、CK7、CK19を標的にして910例について末梢血中および骨髄中ITCの臨床的意義を明らかにした。その結果stage Iからstage IVに至るまで、末梢血液および骨髄においてCEA、CK19またはCK7のうち少なくとも１種類は30-40%の発現を認めた。われわれはRT-PCR法でしばしば問題となる偽陽性あるいは偽陰性について最大限の善処をしている。

2)真の転移能を有するITC検出について

(1)先述した様に、胃癌・乳癌におけるITCの臨床的意義について解析を行い、２種類の癌で相反する結果が得られた。われわれはRT-PCR法でしばしば問題となる偽陽性あるいは偽陰性について最大限の善処をしているが、この結果は乳癌では転移能を有する癌細胞を検出できたのに対し、胃癌では単に循環血中の癌細胞を検出したのみで、転移能を有する癌細胞を検出し得てない可能性を示しているのではないかと考えられる。

他方、白血病において存在が指摘されてきた癌幹細胞が固形癌にも存在する可能性が報告され始めた。癌幹細胞は、Gliomaにおける報告のように転移形成能が強い可能性が示唆されているため、われわれは乳癌と消化器癌で癌幹細胞が存在するか検討を行った。その結果、乳癌では特異的表面マーカーが存在し比較的容易に癌幹細胞が同定できるのに対し、消化器癌ではその同定が困難であることが示された。われわれは真の転移能を有すると考えられる『癌幹細胞様』細胞（SP細胞）を胃癌、大腸癌、食道癌、乳癌において採取した。

(2)骨髄液より癌細胞の単離培養を施行し、増殖能を明らかにした。乳癌、胃癌、大腸癌患者の骨髄を採取し、一定条件のもとに培養を行った。現在、個々の培養増殖能と再発予後との関係を明らかにしている。

【D】考察

特に1)の結果から、癌腫の違いによりITC検出の臨床的意義が異なる可能性が示唆された。

しかしながら、乳癌症例で解析可能であった症例は855例であったが、実際は1500例を越える症例を集積し、PCRでの発現を確認している。さらに、1年間の経過観察期間を設けることで、遊離癌細胞の臨床的意義が明らかになることが期待される。今後、さらなる症例数の増加と経過観察期間の延長が必要である。

一方、胃癌に関しては、上皮系マーカー以外に真の転移能を示唆するマーカーを明らかにする必要があり、このためにおこなっている、2)または3)の実験結果が待たれる。

【E】結論

乳癌では、上皮系細胞の存在自体が予後を予測しうることが示唆された。一方、胃癌では、真の転移能を示唆する新たなマーカーを同定し、再解析を行う必要性が示された。

【F】健康危険情報

特になし

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Detection of occult cancer cells in peripheral blood and bone marrow by quantitative RT-PCR assay for cytokeratin-7 in breast cancer patients

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Abstract. The clinical significance of occult micrometastasis (O.M) remains unknown. We investigated it in peripheral blood (P.B.) and bone marrow (B.M.) in breast cancer patients with surgery. First, we investigated the expression levels of 7 representative molecular markers for detecting O.M (CEA, CK-7, CK-18, CK-19, CK-20, MAM and MUC-1) in 27 cancer and 8 non-epithelial cell lines using quantitative RT-PCR (QRT-PCR), and showed that the expression level of CK-7 was higher in every cancer cell line than in the non-epithelial cell lines. Next, we studied the clinical significance of O.M in P.B. and B.M. by QRT-PCR for CK-7 in breast cancer patients with surgery. Based on comparison with 17 non-cancer controls, 37 (18.0%) and 100 (48.5%) of the 206 patients were positive for CK-7 in P.B. and B.M., respectively. In 98 cases observed over 24 months after surgery, the CK-7-positive group in P.B. had poorer disease-free survival (DFS) than the negative group ($p<0.01$). The CK-7-positive group in P.B. showed poorer DFS than the negative group in 132 lymph node-negative cases ($p=0.01$), and moreover, in 61 lymph node-negative cases observed over 24 months after surgery, the CK-7-positive group in P.B. showed poorer DFS than the negative group ($p<0.0001$). In B.M., no significant difference in DFS was found between the CK-7-positive and CK-7-negative groups. QRT-PCR for CK-7 could be a useful and universal method for detecting O.M, and the quantitative detection of CK-7 in P.B. would have a prognostic value as a marker of early recurrence in breast cancer patients with surgery.

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Abbreviations: QRT-PCR, quantitative reverse transcription-polymerase chain reaction; P.B., peripheral blood; B.M., bone marrow; CEA, carcinoembryonic antigen; CK, cytokeratin; MAM, mammaglobin; MUC-1, mucin-1; ER, estrogen receptor; PgR, progesterone receptor; P.B.M.C, peripheral blood mononuclear cell

Key words: cytokeratin-7, occult micrometastasis, peripheral blood, bone marrow, breast cancer

Introduction

Breast cancer is the most common malignancy of women worldwide, and the incidence of mortality is increasing in Japan (1,2). At the time of primary diagnosis of breast cancer, several clinicopathological parameters such as lymph node status, tumor size, histological grade, and hormone receptor status are used to determine the prognosis and the individual treatment options (3). However, for example, after a 10-year follow-up of breast cancer patients, 15-20% of the patients with lymph node-negative tumors may develop distant metastases (4), whereas approximately 40% of the patients with lymph node-positive tumors may be free of disease and alive for ≥10 years (5,6). Occult micrometastasis in lymph nodes, blood, bone marrow, etc., which is usually missed by conventional tumor staging, is considered to be a determinant of subsequent formation of metastases. The search for occult cancer cells in patients with curatively resected tumors is of considerable importance, because early dissemination of tumor cells is one of the leading causes of relapse at distant sites (7) and of death from cancer (8). Over the years, investigators have attempted to improve techniques for detecting occult metastatic cells, and to clarify the clinical significance of such occult metastases, which may help to predict the prognosis, assess the response to systemic therapy, provide early warning of recurrence and improve survival through timely therapeutic intervention. Several groups, including our group (9-14), have therefore designed sensitive immunocytochemical and molecular assays such as RT-PCR to identify occult cancer cells, and have reported their clinical significance (15).

However, the clinical and prognostic significance of occult micrometastasis, especially, in blood and bone marrow, in which the existence of cancer cells indicates systemic hematogenous spread, is not yet fully understood. Substantial methodological variation (immunohistochemistry, conventional qualitative RT-PCR, etc.) resulted in detection rates varying from 4% to 48% in multiple similar studies (16), and was the predominant reason accounting for the discrepancies among some clinical follow-up studies. Interlaboratory differences in the techniques used and the absence of large studies correlating occult disease with clinical outcome are partially responsible for this uncertainty (17). Standardization of the technique for detecting occult cancer cells, combined with large prospective clinical studies, will be required to clarify the clinical significance of occult micrometastasis.

Quantitative RT-PCR (QRT-PCR) is a new technology that utilizes an online fluorescence detection system, and is used to precisely quantify gene expression levels (18). This high-throughput, highly automated technology allows for the sensitive detection and quantitation of gene expression and is quickly becoming recognized as the technology of choice for the precise measurement of gene expression levels (19). Therefore, we thought that QRT-PCR was most useful for detecting occult cancer cells as a routine test.

We designed a large-scale study using QRT-PCR with LightCycler (Roche Diagnostics) and have been carrying out this study since August 2000 to clarify the clinical significance of occult micrometastasis in peripheral blood (P.B.) and bone marrow (B.M.) of Japanese breast cancer patients. First, we investigated the expression levels of 7 representative molecular markers for detecting cancer cells in 35 cancer or non-cancer cell lines using QRT-PCR, and identified cytokeratin (CK)-7 as the most useful and universal marker for detecting occult cancer cells. Next, we studied the clinical significance of occult cancer cells in P.B. and B.M. by QRT-PCR for CK-7 in 206 breast cancer patients with surgery and 17 non-cancer patients, and the results suggested that CK-7-positive cells in P.B. were related to early recurrence in breast cancer patients with surgery.

Materials and methods

Cell lines. Thirty-five human cell lines: 6 breast cancer, 11 gastric cancer, 10 colorectal cancer and 8 non-epithelial tumor or normal cell lines were used in this study. The cell lines were: no. 1, MCF-7; 2, Mrknu1; 3, YMB1e; 4, CRL1500; 5, SKBR3; 6, YMB1; 7, AZ521; 8, KATO3; 9, MKN1; 10, MKN7; 11, MKN28; 12, MKN45; 13, MKN74; 14, NS8; 15, NUGC3; 16, NUGC4; 17, FU97; 18, Colo201; 19, Colo205; 20, CCK81; 21, Colo320DM; 22, DLD1; 23, HT29; 24, LoVo; 25, WiDr; 26, RCM1; 27, LS174T; 28, LB23; 29, MZ2; 30, Raji; 31, Jakat; 32, HT1080; 33, KMST6; 34, HUVEC; and 35, U937. Numbers 1-6 were breast invasive ductal carcinoma cell lines, 7-17 were gastric adenocarcinoma cell lines, 18-27 were colorectal adenocarcinoma cell lines, and 28-35 were lines of non-epithelial tumor cells such as lymphoma or normal cells such as angioendothelial cells. They were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% humidified CO_2 atmosphere. After the cultured cell lines in a state of subconfluency were homogenized, the total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions.

Patients. From August 2000 to October 2002, 356 consecutive patients who had breast cancer and were admitted to the Department of Breast Oncology, National Kyushu Cancer Center (Fukuoka, Japan), were studied. Patients who had distant metastases, or received preoperative therapy or previous treatment for various cancers were excluded from the study. Both P.B. and B.M. samples that could be analyzed were obtained from 206 of these patients. The 206 patients had undergone surgery at the Cancer Center. Control samples were obtained from 17 patients with non-cancer (e.g., cholelithiasis) who had undergone surgery at the Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University (Beppu, Japan) from 2000 to 2002. All patients gave written informed consent to participate in this study, which was approved by the ethics and scientific committees of each institution. The observation period ranged from 4 to 34 months (median 22 months). Postoperative adjuvant therapy was performed according to the St. Gallen Consensus Conference (3). The patients underwent clinical examinations at least every 3 months and annual mammography, and were further tested only if they had symptoms.

Clinical samples. Aspiration of both P.B. and B.M. was conducted immediately prior to operation under general anesthesia. The B.M. aspirate was obtained from the sternum using a B.M. aspiration needle. P.B. was obtained through a venous catheter. The first 1 ml of both P.B. and B.M. was discarded because of possible contamination by epidermal cells. Each 1 ml sample of P.B. or B.M. was immediately mixed with 4 ml of Isogen-LS (Nippon Gene, Toyama, Japan) and stored at -80°C until RNA extraction. Total RNA was extracted according to the Isogen-LS manufacturer's instructions. All the clinical samples obtained in National Kyushu Cancer Center were sent to the Medical Institute of Bioregulation, Kyushu University, and analyzed at the institute without knowledge of the histopathological and clinical results.

Quantitative RT-PCR assay. The reverse transcriptase reaction (RT) was performed as described previously (9,20). In brief, first-strand cDNA was generated from 2.7 μg of total RNA in a 30 μl reaction mixture containing 5 μl 5X RT reaction buffer (BRL, Gaithersburg, MD), 200 μM dNTP, 100 μM solution of random hexadeoxynucleotide mixture, 50 units of Rnasin (Promega, Madison, WI), 2 μl 0.1 M dithiothreithol, and 100 units of Molony leukemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice. PCR amplification for the quantification of CEA, CK-7, CK-18, CK-19, CK-20, MAM, MUC-1 and ß2-microglobin (ß2-mic) mRNAs in the cell lines, P.B. and B.M. was performed using the LightCycler and a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as described previously (21). For the PCR, 1 μl template cDNA was placed into a 19 μl reaction volume

Table I. Primers used for quantitative RT-PCR assay.

Gene	Primer sequence (5'-3')	Amplicon (bp)	Refs.
ß2-mic	TGAGTGCTGTCTCCATGTTTGA TCTGCTCCCCACCTCTAAGTTG	88	23
CEA	TAAGTGTTGACCACAGCGACCC GTTCCCATCAATCAGCCAAGAA	167	23
CK-7	TGAATGATGAGATCAACTTCCTCAG TGTCGGAGATCTGGGACTGC	75	22
CK-18	GAGACGTACAGTCCAGTCCTTGG CCACCTCCCTCAGGCTGTT	86	22
CK-19	CATGAAAGCTGCCTTGGAAGA TGATTCTGCCGCTCACTATCAG	138	23
CK-20	TCCCAGAGCCTTGAGATAGAACTC GTTGGCTAACTGGCTGCTGTAAC	105	22
MAM	CGGATGAAACTCTGAGCAATGT CTGCAGTTCTGTGAGCCAAAG	108	23
MUC-1	ACCATCCTATGAGCGAGTACC GCCACCATTACCTGCAGAAAC	107	23

containing the reagent, 4.0 mM $MgCl_2$, 0.8 µM of primers and in a LightCycler capillary. The primers are listed in Table I. All of them are intron-overlapping primer sets (22,23). The amplification conditions consisted of 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and elongation at 72°C for 10 sec. After the amplification, the products were subjected to a temperature gradient from 68°C to 95°C at 0.2°C/sec with continuous fluorescence monitoring to produce the melting curve of the products. After proportional background adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was distinguished from the background, and that cycle number was used as a crossing-point value. A standard curve was produced by measuring the crossing point of each standard value (4-fold serially diluted cDNAs of YMB1), and plotting them against the logarithmic value of concentrations. The concentration of each sample was then calculated by setting its crossing points to the standard curve. The expression levels were normalized by ß2-mic, because it was ubiquitously expressed and no pseudogenes for it have been described so far (23). The expression levels were expressed as the values relative to the expression level of YMB1; 10.0. The analysis was performed three times and the averages were calculated.

Statistical analysis. For statistical analyses, StatView-J 5.0 software (SAS Institute Inc., Cary, NC) was used. Associations between the variables were tested by the Fisher's exact test. Survival curves were drawn according to the Kaplan-Meier method and the survival analysis was carried out by the log-rank test. Univariate and multivariate analysis to determine the patient prognosis was performed with Cox regression analysis with the backward stepwise model. All differences were deemed significant at the level of $p<0.05$. The stage and grade of the tumor were classified according to the tumor-node-metastasis classification of the Union International Contre le Cancer (24).

Results

Expression of 7 molecular markers in breast and gastrointestinal cancer cell lines. The quantitative expression levels obtained for the various marker genes are shown in Fig. 1. Maximum expression level of each marker in non-epithelial cell lines was set as the cut-off value for the marker's expression. CK-7, CK-18 and CK-19 were positive in all breast cancer cell lines. Only CK-7 was positive in all cancer cell lines. We considered that CK-7 was the most useful and universal of the 7 representative molecular markers for detecting occult cancer cells using QRT-PCR, and we therefore investigated the clinical significance of occult cancer cells in breast cancer using QRT-PCR for CK-7.

In order to estimate the sensitivity of CK-7 QRT-PCR assay, serial dilution experiments were carried out. YMB1 cells were mixed with peripheral blood mononuclear cells (P.B.M.Cs) from healthy blood donors in a cell ratio ranging from 1:10 to $1:10^6$. This assay was capable of detecting one YMB1 cell among 10^5 P.B.M.Cs (data not shown).

CK-7 expression in P.B. and B.M. of breast cancer patients with surgery. The levels of CK-7 in P.B. and B.M. of both

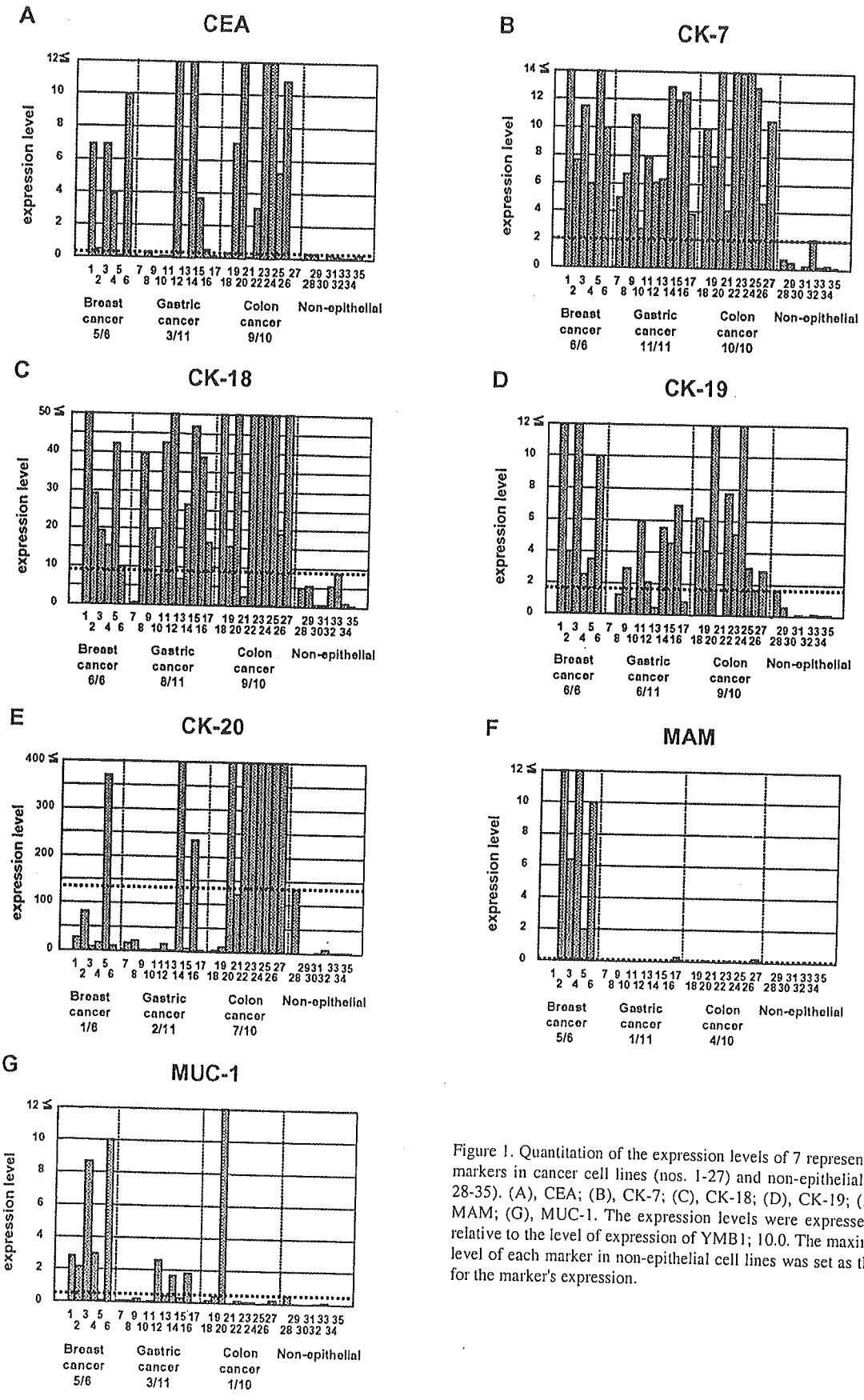

Figure 1. Quantitation of the expression levels of 7 representative molecular markers in cancer cell lines (nos. 1-27) and non-epithelial cell lines (nos. 28-35). (A), CEA; (B), CK-7; (C), CK-18; (D), CK-19; (E), CK-20; (F), MAM; (G), MUC-1. The expression levels were expressed as the values relative to the level of expression of YMB1; 10.0. The maximum expression level of each marker in non-epithelial cell lines was set as the cut-off value for the marker's expression.

the 206 breast cancer patients and 17 controls are plotted in Fig. 2. Among the 206 cases of breast cancer, the mean corrected CK-7 levels (CK-7 mRNA/ß2-mic mRNA ratio) ranged from 0 to 23.0 (average, 1.5; median, 0.8) for P.B. and 0 to 7.9 (average, 1.7; median, 1.4) for B.M. Among the 17 control cases, the mean corrected CK-7 levels ranged from 0.2 to 2.4 (average, 1.1; median, 0.9) for P.B. and 0 to 1.4 (average, 0.7; median, 0.6) for B.M. The maximum expression

Table II. Patient characteristics and detection of CK-7-positive cells in peripheral blood and bone marrow.

Factors	Peripheral blood		p-value[a]	Bone marrow		p-value[a]
	CK-7$^-$ (n=169)	CK-7$^+$ (n=37)		CK-7$^-$ (n=106)	CK-7$^+$ (n=100)	
Age	55.1±11.7 (28-85)	55.6±9.7 (31-75)	NS	55.6±10.8 (30-79)	54.7±12.0 (28-85)	NS
Menopausal status						
Premenopausal	57	12		35	34	
Postmenopausal	100	25	NS	66	59	NS
Unknown	12	0		5	7	
Tumor size[b]						
≤2 cm	47	7		33	21	
2.1-5 cm	102	24		59	67	
≥5.1 cm	20	6	NS	14	12	NS
Lymph node metastasis						
Negative	103	29		73	59	
Positive	66	8	NS	33	41	NS
Lymphatic involvement						
Negative	99	27		62	64	
Positive	70	10	NS	44	36	NS
Vascular involvement						
Negative	165	35		102	98	
Positive	4	2	NS	4	2	NS
Histological grade						
1	21	6		11	16	
2	102	20		65	57	
3	40	9	NS	28	21	NS
Unknown	6	2		2	6	
ER						
Negative	50	11		31	30	
Positive	119	26	NS	75	70	NS
PgR						
Negative	53	18		37	34	
Positive	111	19	NS	64	66	NS
Unknown	5	0		5	0	
Postoperative adjuvant therapy						
Absent	10	6		8	8	
Present	159	31	<0.05	98	92	NS

[a]Correlation was analyzed by Fisher's exact test. [b]Tumor size was measured for invasive area by histological examination. NS, not significant.

level of the control samples (P.B., 2.4; B.M., 1.4) was set as the cut-off value for CK-7-positive cases. Using the respective cut-off values, 37 (18.0%) and 100 (48.5%) of 206 patients were estimated to be positive for the CK-7 in P.B. and B.M., respectively.

CK-7 expression and clinicopathological factors of breast cancer patients with surgery. The correlations between the results for the CK-7 level and clinicopathological factors are summarized in Table II. For both P.B. and B.M., no significant differences in clinicopathological factors such as age, meno-

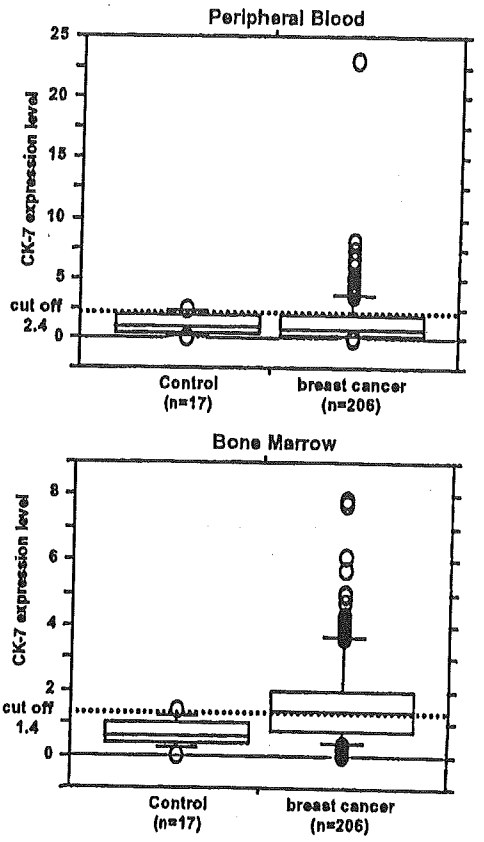

Figure 2. Quantitation of the expression levels of CK-7 in P.B. and B.M. of 206 breast cancer patients with surgery and 17 controls. Top, P.B; bottom, B.M. Median CK-7 expression levels (P.B., 0.9 and 0.8; B.M., 0.6 and 1.4, in controls and breast cancer, respectively) are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quatiles. The expression levels were expressed as the values relative to the level of expression of YMB1; 10.0. The maximum expression level of control samples (P.B., 2.4; B.M., 1.4) was set as the cut-off value of CK-7-positive cases.

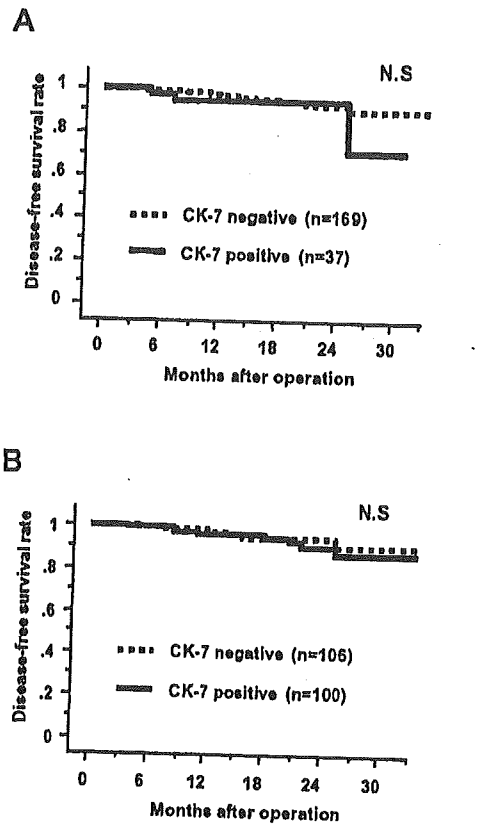

Figure 3. The disease-free survival of 206 breast cancer patients with surgery according to the CK-7 QRT-PCR results. (A), P.B. (B), B.M. In both P.B. and B.M., no significant difference in prognosis was found between the CK-7-positive and CK-7-negative groups.

pausal status, tumor size, lymph node metastasis, lymphatic or vascular involvement, histological grade, ER, and PgR were found between the CK-7-positive and CK-7-negative groups. A significant difference in postoperative adjuvant therapy was found between the CK-7-positive and CK-7-negative groups (Table II; $p<0.05$).

CK-7 expression and disease-free survival (DFS) of breast cancer patients with surgery. First, for all 206 breast cancer patients, DFS was analyzed as shown in Fig. 3. For both P.B. and B.M., no significant difference in prognosis was found between the CK-7-positive and CK-7-negative groups. Then, we evaluated the prognostic significance of occult micrometastasis in 98 cases observed over 24 months because the follow-up period of the other 108 cases was very short. In the 98 cases observed over 24 months after surgery, the CK-7-positive group had poorer DFS than the negative group in P.B. (Fig. 4; $p<0.01$). Univariate Cox regression analysis for DFS revealed that the positive CK-7 in P.B. was a significant risk factor for early recurrence, and other risk factors such as lymph node metastasis, lymphatic involvement, negative estrogen receptor status were also found to be significant.

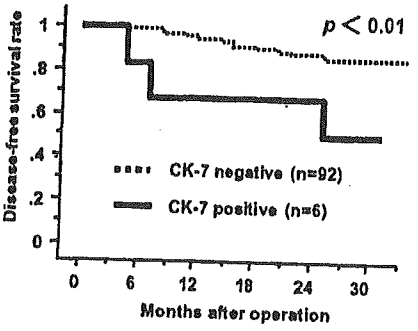

Figure 4. The disease-free survival of patients observed over 24 months after surgery according to CK-7 QRT-PCR results in P.B. Among the 98 cases, the CK-7-positive group showed significantly poorer prognosis than the negative group ($p<0.01$).

Then, multivariate Cox regression analysis (backward stepwise model) for DFS was carried out to assess the independency of each risk factor from others. On the multivariate analysis, tumor size, lymph node metastasis, lymphatic involvement, histological grade, estrogen receptor, CK-7 in P.B. and CK-7 in B.M. were included for the parameters. This analysis demonstrated that the positive CK-7 in P.B. was an independent prognostic factor (Table III). Next, DFS was analyzed for the 132 lymph node-negative patients. In the P.B., the CK-7-

Table III. Prognostic significance of CK-7 QRT-PCR results of 98 patients observed over 24 months after surgery.

Factors		Univariate analysis		Multivariate analysis[a]	
		Relative risk of DFS (95% CI)	p-value	Relative risk of DFS (95% CI)	p-value
Tumor size (cm)	>5/≤5	2.56 (0.83-7.95)	NS	-	NS
Lymph node metastasis	+/-	3.07 (1.11-8.45)	0.03	7.35 (1.99-27.11)	0.003
Lymphatic involvement	+/-	2.92 (1.06-8.04)	0.04	-	NS
Histological grade	3/1, 2	2.19 (0.80-6.03)	NS	-	NS
Estrogen receptor	-/+	2.9 (1.09-7.72)	0.03	5.34 (1.80-15.82)	0.003
CK-7 in P.B.	+/-	4.57 (1.30-16.08)	0.02	27.25 (4.75-156.41)	<0.001
CK-7 in B.M.	+/-	1.60 (0.60-4.26)	NS	-	NS

[a]Seven factors were analyzed by the multivariate method with the backward stepwise model. NS, not significant; CI, confidence interval.

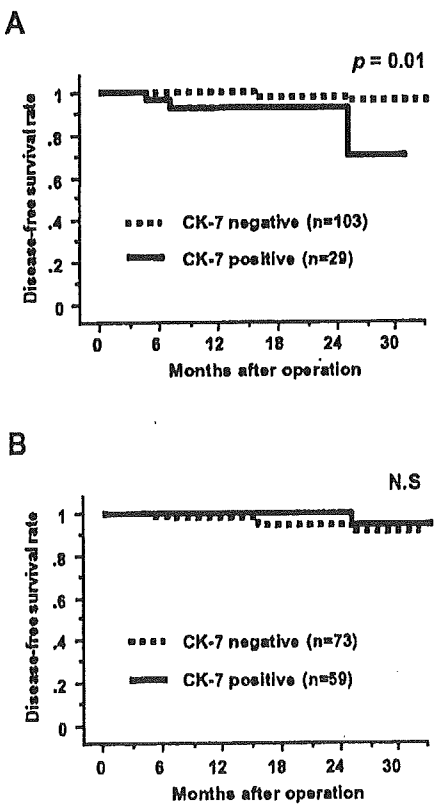

Figure 5. The disease-free survival of 132 lymph node-negative cases according to the CK-7 QRT-PCR results. (A), P.B. (B), B.M. In P.B., the CK-7-positive group showed significantly poorer prognosis than the negative group (p=0.01). In B.M., no significant difference in prognosis was found between the CK-7-positive and CK-7-negative groups.

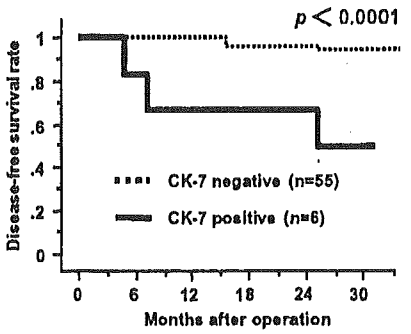

Figure 6. The disease-free survival of lymph node-negative cases observed over 24 months after surgery according to CK-7 QRT-PCR results in P.B. Among the 61 cases, the CK-7-positive group showed significantly poorer prognosis than the negative group (p<0.0001).

positive group had poorer prognosis than the negative group (Fig. 5A; p=0.01). Moreover, in the 61 lymph node-negative cases observed over 24 months after surgery, the CK-7-positive group had poorer prognosis than the negative group (Fig. 6, p<0.0001). In the B.M., no significant difference in prognosis was found between the CK-7-positive and CK-7-negative groups (Fig. 5B). Of the 206 patients, 16 developed recurrence within the follow-up period in this study. Six of the 16 cases were lymph node-negative at the surgery. Four of 6 cases who had distant metastasis (lung, liver, brain or bone metastasis) were CK-7-positive 3 in P.B. and 1 in B.M.

Discussion

The concept of occult micrometastasis has been recognised for several decades, and occult cancer cells have been reported to have proliferative potential *in vitro* (25) and tumorigeneity in nude mice (26,27). There are several ongoing large-scale clinical trials to clarify the clinical significance of occult cancer cells. The American College of Surgeons Oncology (ASCOG) is currently conducting a national trial (protocol Z0010) that will determine the prognostic significance of sentinel node and B.M. micrometastases in women with clinical T1 or T2 N0M0 breast cancer using immunocytochemistry for CKs (28).

RT-PCR assays are more sensitive and objective than immunocytochemistry for the detection of occult micrometastasis in P.B. and B.M. (29-32). However, there has been controversy as to the specificity of the RT-PCR assays.

Some reports showed false-positive results that were thought to be caused by amplification of low-level, illegitimately transcribed target genes (CK-19 or CEA) from hematopoietic cells (33,34). Development of a QRT-PCR assay could solve this problem by setting a cut-off value obtained for control samples so as to differentiate illegitimate target gene expression and cancer-specific expression.

A QRT-PCR assay with high sensitivity, objectivity, preciseness and reproduction, seems to be the method of choice because such a method would be more suitable for routine testing than conventional qualitative RT-PCR. Several groups have attempted to detect occult micrometastasis of breast cancer in the P.B. or B.M. by this method using various markers (35-38). We also have attempted to develop QRT-PCR to study the clinical significance of occult P.B. and B.M. micrometastasis on a large scale.

Various molecular markers have been used to detect occult cancer cells by RT-PCR (39). First, we thought it important to select the most useful marker to detect occult cancer cells by QRT-PCR. It should be noted that the expression level of CK-7 was higher in all cancer cell lines than in non-epithelial cell lines. Other studies have shown that most colorectal cancers did not express CK-7 protein with immunohistochemistry (40). One of the reasons for the discrepancy is that the expression level of CK-7 in colorectal cancer is lower than that in other cancers. CK-7 expression in colorectal cancer may not be detectable by immunohistochemistry but may be detectable by QRT-PCR. CK-7 is one of the low molecular weight cytokeratins whose anatomic distribution is generally restricted to epithelia and their neoplasms (41,42) and is reported to be strongly expressed in breast ductal carcinoma (37,40,43) and gastrointestinal carcinoma (22). Moreover, although coamplification of pseudogenes was previously demonstrated to be a major source of false positivity in RT-PCR, there are no pseudogenes of CK-7 that have been described to date (22). However, Felton *et al* demonstrated that CK-7 was detected in normal peripheral blood by qualitative RT-PCR (44). CK-7, like CK-19 or CEA, might be induced by inflammation and various cytokines. Because background transcription of most of the target genes is unavoidable, quantification of the amplification product and subsequent definition of cut-off values for each target gene is the only way to improve the specificity of tissue-specific RT-PCR assays. In this study, we evaluated several candidate genes, and found that CK-7 was detected in all cancer cell lines examined using QRT-PCR with these cut-off values. Therefore, we considered that CK-7 was the most useful and universal marker for detecting occult metastatic cancer cells using QRT-PCR.

Next, we investigated the relationship between CK-7 expression in P.B. and B.M. and clinicopathological factors in 206 breast cancer patients with surgery. Using the cut-off levels set based on the values in 17 non-cancer controls, 37 (18.0%) and 100 (48.5%) of the 206 patients were positive for CK-7 expression in P.B. and B.M., respectively. Similarly to our results, several groups have reported higher detection rates for occult cancer cells in B.M. than in P.B. (32,45). We found no significant differences between the CK-7-positive and CK-7-negative groups with regard to clinicopathological factors in either P.B. or B.M. This apparent inconsistency may be due to the different dissemination pathways that breast cancer cells utilize, i.e., lymphatic spread of the tumor is independent of hematogenous dissemination. Considering that solid tumors usually contain multiple clones, it is possible that only a small subset of the primary tumor cells have the biologic characteristics that favor dissemination, by analogy to the fact that only a minority of tumor cells in primary tumors have tumorigeneity in immunocompromised mice (46). Therefore, the probability of finding disseminated cancer cells may not necessarily parallel the load of the primary tumor, nor can it be predicted by the well-known risk factors. Remarkably, a significant difference in postoperative adjuvant therapy was found between the CK-7-positive and CK-7-negative groups ($p<0.05$). These data indicate that this QRT-PCR assay of CK-7 in P.B. could identify high-risk patients who were considered low-risk patients by the St. Gallen Consensus Conference.

Moreover, we evaluated the prognostic significance of CK-7 expression in the P.B. and B.M. of the breast cancer patients. In the P.B. of all 206 patients, no significant difference in prognosis was found between CK-7-positive and CK-7-negative groups. However, among 98 cases observed over 24 months after surgery, the CK-7-positive group showed significantly poorer prognosis than the negative group in P.B. ($p<0.01$) and the positive CK-7 in P.B. was an independent prognostic factor from the clinicopathological risk factors. Next, in 132 lymph node-negative cases, the CK-7-positive group in P.B. showed significantly poorer prognosis than the negative group ($p=0.01$). Moreover, in 61 lymph node-negative cases observed over 24 months after surgery, the CK-7-positive group showed significantly poorer prognosis than the negative group ($p<0.0001$). These results suggest that occult cancer cells in P.B. are related to early recurrence in breast cancer patients. Stathopoulou *et al* also reported that molecular detection of CK-19-positive cells in P.B. of breast cancer patients using conventional nested RT-PCR has independent prognostic value as a marker of poor clinical outcome (45). As breast cancer patients with early recurrence within 24 months after the surgery tend to have poorer prognosis, earlier detection of patients who have a high risk of early recurrence is important for offering appropriate treatment with adjuvant therapy. This QRT-PCR for CK-7 in P.B. of breast cancer would be a useful method for detecting such high-risk patients.

In the B.M., no significant difference in prognosis was found between the CK-7-positive and CK-7-negative groups in this observation period. Many studies have shown that the detection of occult cancer cells in the B.M. is associated with an increased risk of recurrence and reduced survival in patients with breast cancer (47). These findings and our results suggest that occult cancer cells in the B.M., most of which are 'dormant' as described by Pantel *et al* (48), are scattered in the P.B. when activated, and become established in internal organs (e.g., bone, brain and lung) hospitable to the cancer cells. After a longer follow-up, CK-7 positivity in B.M. should be evaluated as a prognostic factor.

In this study, we showed that CK-7 was the most useful marker for detecting occult cancer cells by QRT-PCR, and CK-7-positive cells in the P.B. were related to early recurrence in breast cancer with surgery. A QRT-PCR assay for CK-7

could be a powerful and universal method for detecting occult cancer cells in breast cancers. This strategy for detection of occult micrometastasis would help in the design and monitoring of new therapeutic strategies for prevention of metastatic disease recurrence as well as the selection of low-risk breast cancer patients who do not need postoperative adjuvant therapy. Of course, further study in a larger series with a longer follow-up period is needed to conclude the clinical significance of micrometastasis in P.B.

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特集：乳　　癌

微小転移

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基礎研究

微小転移

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Occult micrometastasis

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Abstract

The concept of occult micrometastasis has been recognized for several decades. Recently, the balance of evidence favours the hypothesis that micrometastatic cells impacts on survival in spite of few clinical large-scale trials. Immunocytochemistry using anti-cytokeratin monoclonal antibodies is often used to detect micrometastatic cells. The strategy for detection of micrometastasis would help in the design of "tailor-made treatment". Furthermore, the genomic analyses of micrometastatic cells will contribute to the development of more effective strategies to treat cancer patients as well as the clarification of the carcinogenesis and the mechanism of clinical metastasis which is the leading cause of cancer-related death.

Key words: micrometastasis, cytokeratin, tailor-made treatment, parallel evolution

はじめに

乳癌の診断は，視触診をはじめとしてマンモグラフィや超音波検査，細胞診，マンモトームなどによる局所診断に加え，CTや骨シンチグラムなどによる全身への転移診断により行われ，それらによる進行度評価のうえ治療方針が決定される．そして，手術が施行された場合は，腫瘍の大きさ(T)，リンパ節転移(N)，遠隔転移(M)で規定されるTNM分類を中心に年齢，細胞分化度(grade)，脈管浸潤，ホルモン受容体(ER, PgR)発現，HER2発現などの確立した予後因子の評価を行い術後補助療法などの治療方針が決定されている(St. Gallen Consensus Conference)．TNM stage分類は，進行度，予後を反映し，世界的に広く用いられているが，同一stageであっても予後が異なる症例が少なくなく，また，術後10年以上経過し治癒と考えられた症例でも再発することもまれではない．そこで，①通常行われている検査では検出することが困難(不可能)な微量な癌細胞が存在し，その微量癌細胞が再発や転移に関与するのではないか(微小転移)，②微量癌細胞が再発転移に関与するならば，それを検出することでより詳細な進行度分類とより適した治療を行うことができるのではないか，との想定のもとで微小転

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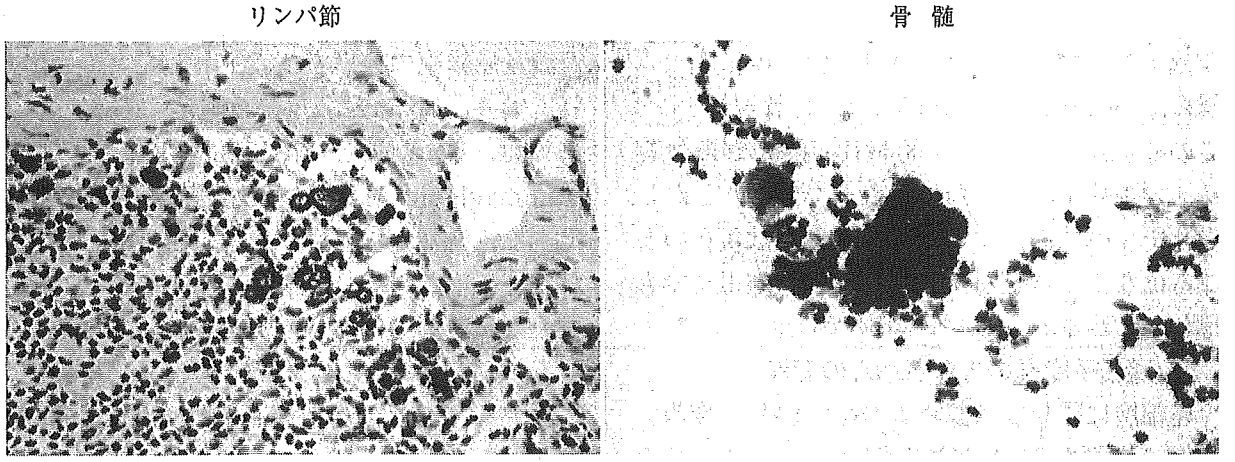

図1 微量癌細胞(免疫染色法)

移研究が進められている.

1. 微量癌細胞(図1)

微量癌細胞とは, 通常行われている検査(画像診断, 腫瘍マーカー, 病理診断)では診断できないごくわずかな癌細胞と定義される. 潜在癌細胞(occult cancer cell)とも表記される. また, 微量癌細胞が存在するものの転移を形成したかどうか不明なisolated tumor cellと転移巣を形成したと考えられる微小転移(micrometastasis)を厳密に区別する主張もある. UICCやAJCCでは, 2mm以下のリンパ節転移をmicrometastasis(pN1a)としている. リンパ節転移は, 最終的には病理組織検査で診断されるが, これは通常, リンパ節の最大割面における転移の有無をH.E染色することにより診断される. よって最大割面に露出しないほどの微量な癌細胞(群)は, ルーチンとしての病理組織診断では検出されないため転移陰性と診断されることになる. また, H.E染色は個々の細胞の形態を観察するには最も適した診断法であるが, 多くの細胞集団の中から少数の癌細胞を検出する方法としては適していない. また, 全身への転移診断として, CTや骨シンチグラム, 最近ではFDG-PETなどの画像診断を行っているが, 少数の癌細胞を検出することは不可能である.

そこで, 一般的には微量癌細胞を検出する方法として, 高感度な検出法, すなわち免疫染色法やRT-PCR法などの分子生物学的手法が用いられている.

2. 微量癌細胞のviability

これまでの基礎的研究によると, 微量癌細胞は, *in vitro*で増殖能を有しており[1], ヌードマウスへ皮下移植すると腫瘤を形成すること[2,3]から, それ自身が転移形成能をもつと考えられる. しかし, 検出された微量癌細胞が転移を成立(微小転移)させるかどうかは不明である. 臨床的には, 転移を形成する悪性度の高い微量癌細胞を検出することが望まれる. そこで癌の悪性度に強く関与すると考えられているuPA(urokinase plasminogen activator)やcathepsin-D, erbB2などの発現を併せて評価することで, 存在診断単独よりも強く予後に相関するとの報告がある[4,5]. また, 微量癌細胞の*in vitro*での増殖能がより臨床的に予後に反映するとの報告もある[1].

3. 微量癌細胞の分子標的マーカー

微量癌細胞を検出するには, 正常細胞にはない癌細胞の特異的変化を検出すればよい. 癌細胞の正常細胞との違いは大きく次の3つに分類される.

(1) 遺伝子変異の有無(DNAの変異)
(2) 遺伝子発現量の変化(mRNA, 蛋白量)
(3) 蛋白構造, 機能の変化(例:酵素活性)