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Identification of differentially expressed genes in isogenic highly metastatic and poorly metastatic cell lines of R3230AC rat mammary adenocarcinoma

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Abstract. Tumour metastasis occurs as a result of a cascade of events including alterations in the expression of various genes. The identification of such genes is essential to understanding formation of metastasis. In a previous study, highly metastatic (LN4.D6) and poorly metastatic (CAb.D5) cell lines were obtained from the rat mammary adenocarcinoma cell line R3230AC. Subtractive hybridization was used to identify differentially expressed genes between these two cell lines. We identified eight cDNA clones in CAb.D5 and six cDNA clones in LN4.D6 that were differentially expressed. One of the cDNA clones in each cell line had no homology with known sequences. Expression patterns of these differentially expressed genes were examined in a pair of rat mammary and prostate adenocarcinoma cell lines. Compared with cell lines examined, cDNA FF-10 was only expressed in CAb.D5; however, cDNA RB-8, RE-1, RF-5 were only expressed in the highly metastatic LN4.D6. No correlation was observed between expression patterns of the differentially expressed genes and metastatic potential of these cells. However, differential expression of genes, especially cytokeratins (CK8 and CK5) and collagens (III and IV) between highly metastatic and low metastatic rat mammary adenocarcinoma cell lines might initiate further investigation of these genes in metastatic process.

INTRODUCTION

Steps in human cancer progression have been well identified clinically. In some cases, premalignant lesions exist in organs before they progress to malignant invasive tumours. These may occur as a result of either genetic alterations or environmental factors. Accumulation of mutations in a cell may convert it into a malignant phenotype which could give rise to a primary tumour. At early stages of primary tumour growth, cells are not invasive nor metastatic. However, further accumulation of genetic alterations results in invasive and metastatic cells being generated (reviewed by Yokota 2000). Metastasis is a multistep process in which tumour cells

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leave their primary location, invade surrounding normal tissue, penetrate into blood and lymphatic vessels, escape host anti-tumour responses and establish secondary cancers in other tissues (Liotta *et al.* 1991). The genetic events controlling this process are not yet well known. Even though there has been significant improvement in diagnosis, most deaths from cancer are still due to metastatic tumours. Therefore, identification of genes and gene products involved in metastasis is the subject of many current studies.

A variety of experimental models is available for the investigation of cancer metastasis. There is substantial evidence that tumours contain phenotypically and biologically heterogeneous cell populations resulting from differences in the genes altered in each cancer cell (Fidler *et al.* 1987). Based on this, a tumour subpopulation from R3230AC rat mammary adenocarcinoma was selected in this laboratory which exhibited enhanced metastatic ability to draining lymph nodes [and measured by lung colony formation after tail vein injection (Carlsen *et al.* 1993)]; it was designated LN4 (Buckly & Carlsen 1988). This cell line was shown to bind the lectin soybean agglutinin via neutral isoglobotetraosylceramide (iGb4Cer) expressed on the cell surface (Buckly & Carlsen 1988; Carlsen *et al.* 1990; Carlsen *et al.* 1993). When the LN_4 cell subpopulation was depleted of cells expressing iGb4Cer by incubation with an anti-iGb4Cer monoclonal antibody (mAb), the resulting cell line CAb exhibited low metastatic activity (Carlsen *et al.* 1990). Recent studies have shown that expression of iGb4Cer in highly metastatic cells results from a significant decrease in CMP-NeuAc : lactosylceramideα2,3-sialyltransferase (GM3 synthase) activity (Dumonceaux & Carlsen 2001).

In order to elucidate the mechanism of metastasis in this cell model system, it is important to identify genes that are differentially expressed in highly metastatic versus poorly metastatic cell lines. Subtractive hybridization is a powerful approach to isolate differentially expressed genes (Wang & Brown 1991; Wan & Erlander 1997; Vedoy *et al.* 1999). Therefore, here, we have used this method along with differential screening to identify genes that were differentially expressed in highly metastatic (LN4.D6) and poorly metastatic (CAb.D5) cell lines. We found that eight forward cDNA clones in CAb.D5 and six reverse cDNA clones in LN4.D6 cell lines were differentially expressed compared with each other. However, no correlation was found between the expression patterns of the differentially expressed genes and metastasis in highly metastatic LN4.D6 compared with MAT.LyLu cell line and poorly metastatic CAb.D5 and the non-metastatic AT1 cell line.

MATERIALS AND METHODS

Cells and culture conditions

LN4.D6 and CAb.D5 were both clones derived from the highly metastatic LN4 and poorly metastaic CAb R3230AC cell lines, respectively (Buckly & Carlsen 1988; Carlsen *et al.* 1990; Carlsen *et al.* 1993). Highly metastatic MAT-LyLu and non-metastatic AT1 rat prostate adenocarcinoma cell lines were obtained from European Collection of Cell Cultures (ECACC). All cell lines, including a normal rat fibroblast cell line, Rat.2 from ECACC were maintained in RPMI 1640 medium supplemented with 10% calf serum, penicillin (100 U/ml) and streptomycin sulphate (100 mg/ml). The same medium contained 200 mM glutamine and 100 mM dexamethasone in order to improve the plating efficiency of MAT.LyLu and AT1 cell lines. Cells were incubated at 37 °C in 5% CO_2, 95% air in a humidified incubator. When they reached 60–80% confluence, they were collected for RNA isolation.

RNA isolation

Total RNA was isolated from 60 to 80% confluent cells according to the method of Chomczynski & Sacchi (1987). Messenger RNA (mRNA) was isolated from total RNA using oligo (dT) cellulose or NucleoTrap mRNA Purification Kits (Clontech, Palo Alto, CA, USA). Isolated RNA pellets were resuspended in water, quantified by spectrophotometry and stored at −70 °C.

Subtractive hybridization

PCR-Select cDNA subtraction was carried out as described in the Clontech subtractive hybridization kit protocol. Briefly, mRNA (2 μg) from Cab.D5 or LN4.D6 was reverse transcribed to cDNA with cDNA synthesis primer in the presence of 20 units of AMV reverse transcriptase and dNTPs at 42 °C for 1.5 h in an air incubator. After performing second-strand cDNA synthesis at 16 °C for 2 h, double-stranded (ds) cDNA of either Cab.D5 (tester) or LN4.D6 (driver) was cut with *Rsa*I restriction enzyme. Two different adaptors (adaptor 1 or adaptor 2R) were ligated to the *Rsa*I-digested tester cDNA. After analysis of ligation efficiency, Cab.D5 tester cDNA with adaptor 1 or adaptor 2R was mixed with LN4.D6 driver cDNA for first hybridization at 68 °C for 8 h to obtain forward-subtracted cDNA. In order to further enrich for differentially expressed sequences, a second hybridization was carried out overnight at 68 °C. New hybrid molecules with different adaptors on each end were amplified with 50 × Advantage cDNA Polymerase mix (Clontech) in a Perkin Elmer GeneAmp PCR System 2000 thermocycler (27 cycles: 94 °C, 10 s; 66 °C, 30 s; 72 °C, 1.5 min). The same protocol was applied to generate reverse-subtracted cDNA where LN4.D6 served as tester and CAb.D5 was the driver.

Subtracted cDNA library construction and differential screening

PCR products of subtracted cDNA were further amplified using nested primers for adaptor 1 and adaptor 2R with Taq Polymerase (Qiagen, Hilden, Germany) at 18 cycles: 94 °C, 10 s; 68 °C, 30 s; 72 °C, 1.5 min. These secondary PCR products were ligated to pCR2.1 plasmid vector (T/A cloning kit, Invitrogen, San Diego, CA, USA) according to the manufacturer's directions. INV F′ competent cells were transformed with pCR2.1 constructs and transformants selected based on colour formation on Luria broth plates containing ampicillin and X-gal (Sigma, St Louis, MO, USA).

Randomly picked white transformant colonies were grown in a 96-well plate for differential screening. One microlitre of each bacterial culture was transfered to a 96-well plate and amplified with nested primers at 23 cycles; 95 °C, 30 s; 68 °C, 3 min in Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). After verification of each PCR product on a 1.5% agarose ethidium bromide gel, two identical dot blots of PCR products were prepared to hybridize with either forward- or reverse-subtracted cDNA probes. For dot blots, 5 μl of each PCR product was combined with 0.6 N NaOH, and 3 μl of this mixture was then transferred to nylon membrane and cross-linked with UV Stratalinker (Stratagene, La Jolla, CA, USA).

Preparation of cDNA probes and dot blot hybridization

Adaptor sequences of secondary PCR products of forward- or reverse-subtracted cDNA were removed using *Rsa*I, *Eag*I and *Sma*I restriction enzymes. PCR products were purified with Qiaquick PCR purification kit (Qiagen) and labelled with [α-^{32}P]dCTP using random priming as described in PCR-Select Differential Screening kit (Clontech).

Each dot blot was hybridized either with forward- or reverse-subtracted cDNA probe according to the manufacturer's directions (Clontech). Membranes were pre-hybridized in ExpressHyb solution at 72 °C for 60 min and hybridized with [α-^{32}P]dCTP-labelled cDNA probe overnight at 72 °C. Membranes were then washed and exposed to X-Omat AR film (Kodak) at 70 °C for up to 2 days.

Northern blot analysis

Messenger RNA (2.5 μg per lane) was electrophoresed on a 1.2% agarose-formaldehyde gel and then transfered to nylon membrane (Boehringer Mannheim, Mannheim, Germany) by capillary action. Blots were pre-hybridized with ExpressHyb solution (Clonetech) for 1 h, hybridized with probes and labelled with [α-^{32}P]dCTP by random priming for 2 h at 68 °C. Membranes were washed three times with 2 × SSC, 0.2% sodium dodecyl sulfate (SDS) at room temperature for 10 min each, followed by two washes with 2 × SSC, 0.2% SDS at 50 °C for 20 min each. Membranes were exposed to X-Omat AR film (Kodak) or a Molecular Imager screen (Bio-Rad Laboratories, Hercules, CA, USA) for up to 24 h. The sizes of mRNA transcripts were determined by comparison with RNA Marker I (Promega, Madison, WI, USA).

cDNA sequencing

Individual bacterial colonies containing pCR2.1 plasmid vector with differentially expressed cDNA fragments were grown overnight in 2 ml Luria broth supplemented with 100 μg/ml ampicillin (Sigma). Plasmid DNA was isolated by a rapid alkaline lysis method and electrophoresed on a 1% agarose gel. Plasmids containing cDNA inserts were sequenced with an automated sequencer (310 Genetic Analyser, ABI Prism). DNA sequences were analysed for homologies to known DNA/mRNA sequences through BLAST search of GeneBank Database at the National Center for Biotechnology Information (National Institute of Health, Bethesda, MD, USA).

RESULTS

Subtractive hybridization

Subtractive hybridization was used in order to determine differentially expressed genes in the highly metastatic LN4.D6 and poorly metastatic CAb.D5 cells. An outline of this approach is shown in Fig. 1. Subtractive hybridization consists of generating double-stranded (ds) cDNA from mRNA of two cell lines which serve as either tester or driver, digesting of tester or driver cDNA with *Rsa*I enzyme to get blunt-ended cDNA, creating two tester populations with different adaptors, and first and second hybridization of tester and driver cDNA to generate differentially expressed sequences. In order to exponentially amplify only differentially expressed sequences, subtracted cDNA was amplified using suppression PCR (Siebert *et al.* 1995) in which a pan-like structure occurs when complementary sequences are present on each end of the single-stranded DNA. During the secondary PCR amplification, the background was reduced and differentially expressed sequences further enriched using nested primers. In this experiment, we generated forward-(CAb.D5 tester; LN4.D6 driver) and reverse-subtracted cDNA (LN4.D6 tester; CAb.D5 driver). Subtraction efficiency was confirmed by reduction of a housekeeping gene G3PDH abundance in forward- or reverse-subtracted cDNA but not in unsubtracted cDNA (data not shown).

Differential screening of forward- and reverse-subtracted clones

When membranes prepared from forward-subtracted cDNA (Fig. 2a and b) were compared with each other, many strong hybridizing dots appeared on the membrane probed with forward-subtracted cDNA (Fig. 2a); however, only one dot was apparent on the membrane probed with reverse-subtracted cDNA (Fig. 2b) indicating that differentially expressed genes in CAb.D5 cells were successfully subtracted. Similarly, comparison of the membranes prepared from reverse-subtracted cDNA (Fig. 2c and d) showed that the membrane probed with reverse-subtracted

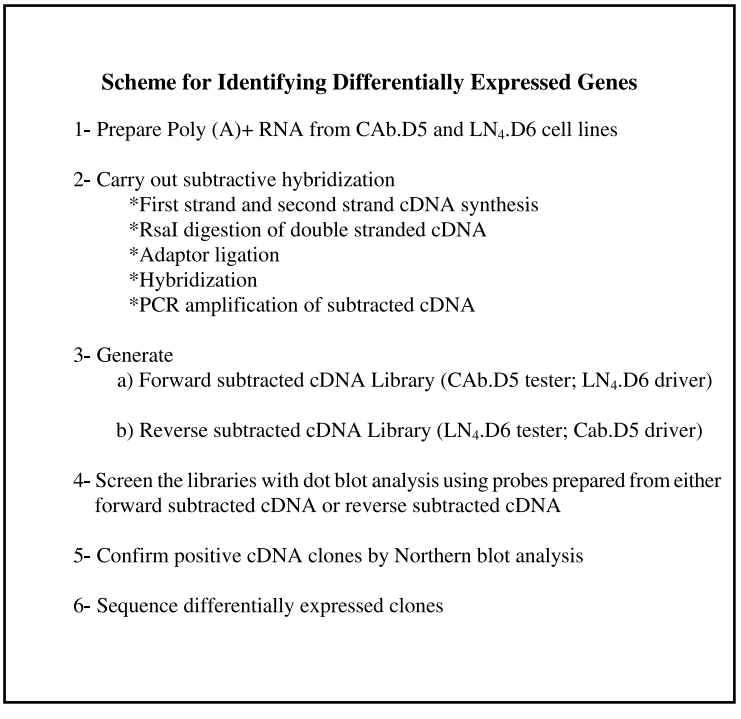

Figure 1. Schematic representation of the procedure for isolation of differentially expressed genes in CAb.D5 and LN_4.D6.

cDNA (Fig. 2c) had many more strongly hybridizing dots than the membrane probed with forward-subtracted cDNA (Fig. 2d).

Twenty-eight clones from forward-subtracted cDNA and 18 clones from reverse-subtracted cDNA were further tested with northern blot analysis for the confirmation of differentially expressed genes in LN4.D6 and CAb.D5 cells lines.

Northern blot and identification of sequences for differentially expressed genes

In order to confirm differential gene expression between LN4.D6 and CAb.D5 cells selected by differential screening, northern blot analysis had been carried out and sizes of mRNA transcripts detected by the cDNA clones were determined.

A total of 28 randomly selected forward-subtracted cDNA clones were screened with northern blotting and 18 of the clones were positive for differential gene expression in CAb.D5 cells; however, 40% of the screened clones were false positive. All positive clones [FA-8, FB-5 (absent in figure), FC-6, FC-7, FF-10, FC-1, FH-2 and FG-6] were observed to be expressed only in CAb.D5 (Fig. 3a) but not in the LN_4.D6 cell line.

In addition, using northern blot analysis, 11 of 18 reverse-subtracted cDNA clones were confirmed to be positive for differential gene expression in the LN_4.D6 cell line (Fig. 3b). The clones RB-8, RB-9, RE-1, RE-12 and RF-5 were expressed only in the LN_4.D6 cell line. In contrast, the RD-4 clone was expressed in both cell lines (only Fig. 3b has it) but the expression level of RD-4 was twofold higher in LN4.D6 than in CAb.D5.

In order to determine if any of the isolated clones were identical or if they corresponded to previously characterized genes, sequencing of the cDNA clones was carried out. Sequence data

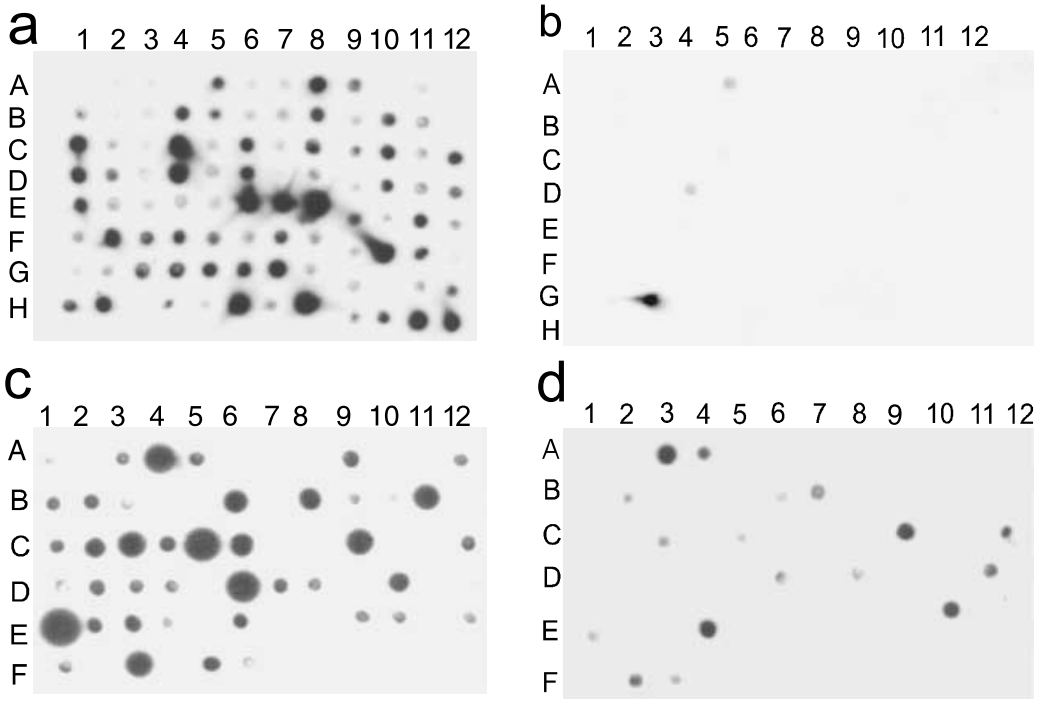

Figure 2. Differential screening of subtracted cDNAs. Randomly chosen bacterial colonies from forward- or reverse-subtracted cDNA library were grown in 96-well plate, amplified with PCR and arrayed on nylon membranes. (a) and (b) are identical membranes for forward-subtracted clones; (c) and (d) for reverse-subtracted clones. The membranes (a) and (d) were hybridized with a forward-subtracted cDNA probe; the membranes (b) and (c) hybridized with a reverse-subtracted cDNA probe. Wells number A-1 and A-4 of membrane (a) served as negative controls for hybridization (testis specific protein cDNA).

of each clone was compared with known sequences using the BLAST homology search program. With this approach, we were able to identify six cDNA clones, FA-8, FC-1, FC-6, FC-7, FF-10, FH-2 which showed homologies to previously sequenced genes, corresponding to, respectively, alpha 2 collagen IV, aldehyde dehydrogenase, pro-alpha 1 collagen III, ribosomal protein L4, osteoblast/osteocyte factor 45, and cytokeratin 8. The remaining one cDNA clone FG-6 presented no homologies with known sequences (Table 1).

The same analysis was carried out for differentially expressed cDNA clones in the LN_4.D6 cell line and five cDNA clones were identified as having homologies to known genes (Table 1). However, the cDNA RE-12 had no sequence match in the BLAST. Full-length cDNA-sequence analysis of the novel genes is under investigation.

Expression profile of differentially expressed genes in different cell lines

Northern blot analysis was performed to determine whether there was a correlation between the expression profile of differentially expressed genes and metastasis in two pairs of rat adenocarcinoma cell lines with low and high metastatic potential. Even though forward-subtracted genes were differentially expressed in CAb.D5 cells compared with LN_4.D6 cells, the expression pattern of these genes in CAb.D5 and LN_4.D6 did not show any similarity to that of the genes of the rat prostate MAT-LyLu and AT1 cells (Fig. 4, Table 2). However, only the expression

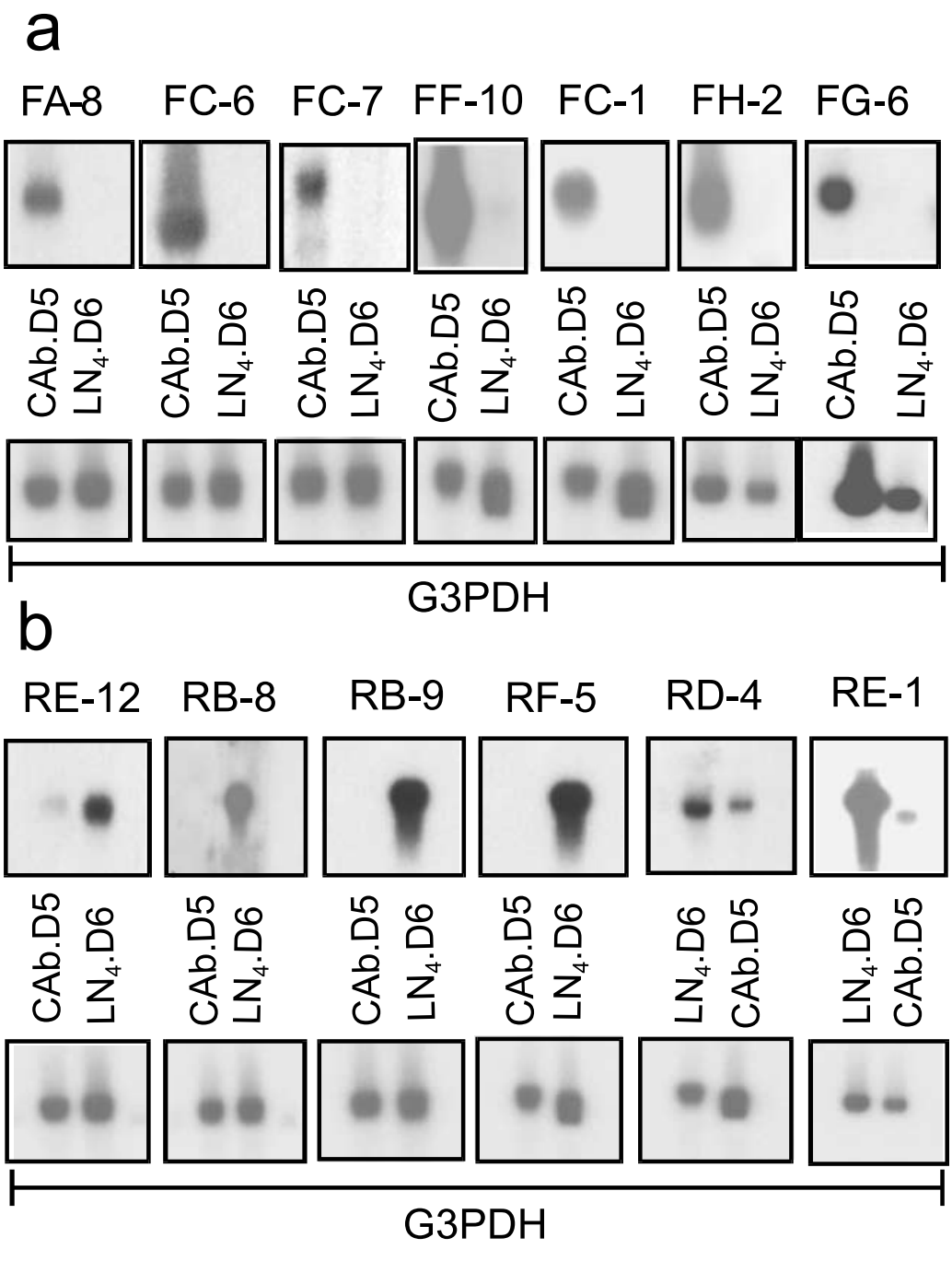

Figure 3. Northern blot analysis of forward- and reverse-subtracted cDNA clones. Each membrane containing mRNA for CAb.D5 and LN_4.D6 cell lines was probed with a different cDNA clone. Membranes in (a) were probed with forward-subtracted cDNAs, membranes in (b) were probed with reverse-subtracted cDNAs. For the confirmation of mRNA in both lanes, the membranes were stripped and labelled with G3PDH internal control.

Table 1. Differentially expressed cDNA clones in higly metastatic LN_4.D6 and poorly metastatic CAb.D5 cell lines

Clone	Number of sequenced nucleotides	Size of mRNA (kb)	Sequence	% homology	Number of clones
FA-8	236	2.7	Rat alpha 2 collagen IV	87	1
FB-5	–	2	ND		1
FC-1	360	1.6	Rat aldehyde dehydrogenase	99	1
FC-6	226	4.6	Rat proalpha 1 collagen III	92	1
FC-7	218	0.8	Ribosomal protein L4	93	1
FF-10	463	1.3	Osteoblast/osteocyte Factor 45	95	1
FG-6	282	0.5	Novel		3
FH-2	297	1.8	Rat cytokeratin 8	95	9
RB-8	536	1	Neutrophil chemoattractant 2	95	2
RB-9	410	1.6	Human LGN protein	85	1
RD-4	88	1.3	Mouse aspartylaminopeptidase	89	1
RE-1	417	1.6	Rat keratin (C K5) mRNA	97	3
RE-12	361	1	Novel		1
RF-5	321	1.4	Mouse keratin (epidermal) type 1	95	3

cDNA clones were obtained by subtractive hybridization of CAb.D5 and LN_4.D6 cells. Forward- and reverse-subtracted clones are designated as FA-8 to FH-2; and RB-8 to RF-5, respectively. ND, not determined.

Table 2. Relative expression levels of mRNA for differentially expressed genes in different cell lines

Clones	Cell lines				
	LN_4.D6	CAb.D5	MAT-LyLu	AT1	Rat.2
FA-8	–	242	140	210	60
FB-5	ND	ND	ND	ND	ND
FC-1	–	121	157	147	140
FC-6	–	232	93	52	24
FC-7	110	233	168	183	190
FF-10	–	120	–	–	–
FG-6	80	190	150	166	320
FH-2	–	215	166	113	47
RE-1	150	–	–	–	–
RB-9	206	113	140	180	280
RF-5	252	–	–	–	–
RD-4	145	95	145	190	460
RB-8	235	–	–	–	–
RE-12	130	60	30	70	80

Expression levels of differentially expressed forward- and reverse-subtracted genes was determined by northern blot analysis. Level of mRNA was quantified with phosphor imager and normalized with the level of G3PDH. Identity of forward- and reverse-subtracted clones are indicated in Table 1.
ND, not determined.

profile of FA-8 had some similarity in the two pairs of cell lines, in that FA-8 gene expression was high in poorly metastatic CAb.D5 and non-metastatic AT1 cell lines compared with highly metastatic LN4.D6 and MAT-LyLu cell lines (Table 2). In addition, FF-10 gene expression was observed only in CAb.D5 cells (Fig. 4). Except FF-10, all forward-subtracted cDNA clones were expressed in Rat.2 cell line (Table 2). Moreover, the expression level of FG-6 was the highest in Rat.2 cell line compared with the other cell lines.

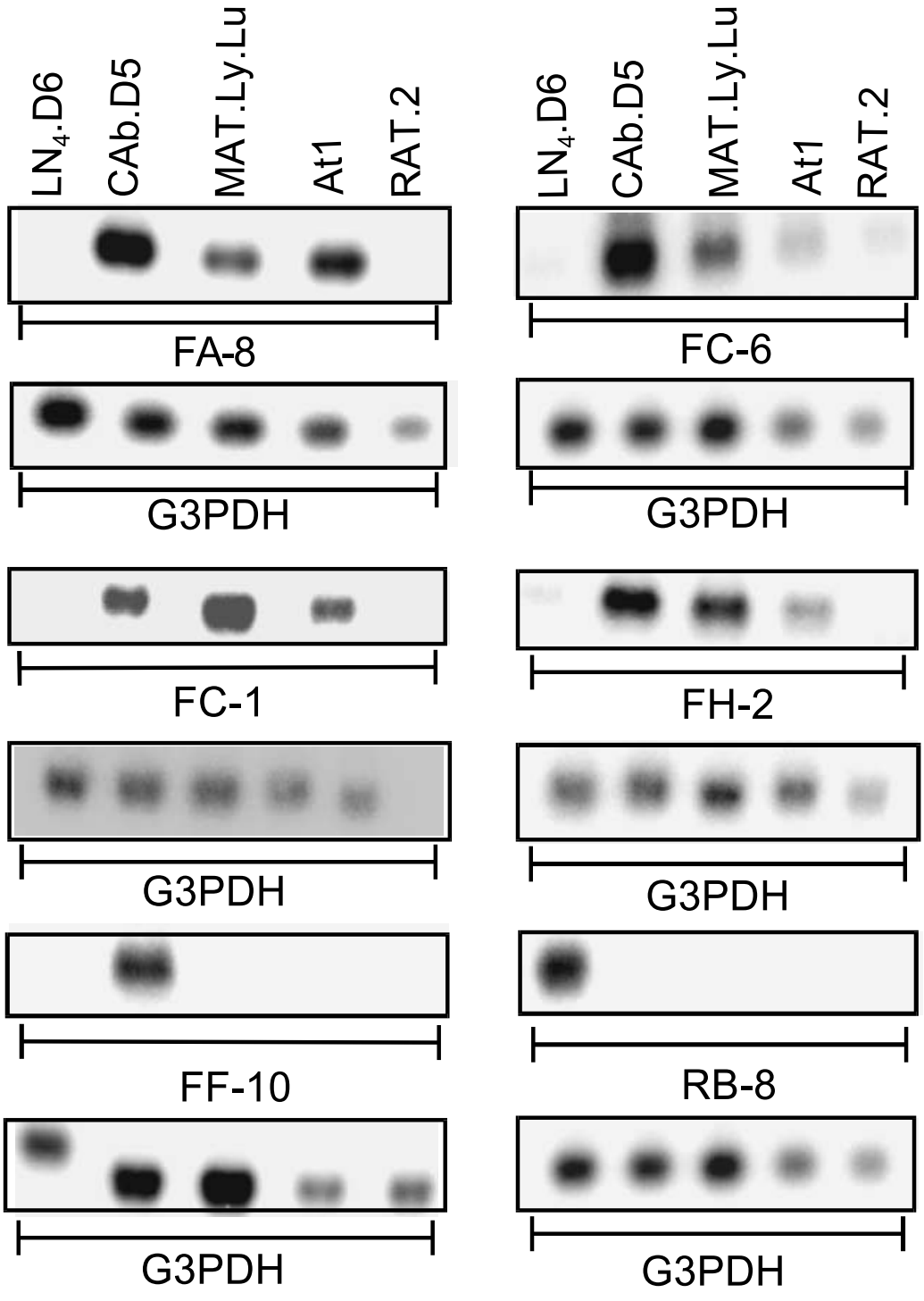

Figure 4. Expression profile of differentially expressed genes in different cell lines. Northern blot membranes of FA-8, FC-6, FC-1, FH-2, FF-10 and RB-8 were stripped and labelled with G3PDH as an internal control.

Similarly, the expression pattern of differentially expressed reverse-subtracted genes in LN4.D6 and CAb.D5 did not correlate with that of the genes in MAT-LyLu and AT1 cell lines (Table 2). In addition, RB-8 (Fig. 4), RE-1, and RF-5 were exclusively expressed in LN4.D6 (Table 2). Finally, the expression level of RD-4 clone was almost threefold higher in Rat.2 cell line compared with other cell lines (Table 2).

DISCUSSION

Cancer metastasis is regulated by the activation and inactivation of many genes in different cancer types (Ahmad & Hard 1997; Yokota 2000). It is possible that each step of metastasis is controlled by transient and/or permanent changes at the mRNA level of different genes. Therefore, in the present study, we investigated differentially expressed genes in highly metastatic (LN4.D6) and poorly metastatic (CAb.D5) subpopulations of R3230AC rat mammary adenocarcinoma cells using a subtractive hybridization technique which enables efficient and rapid cloning of even rarely transcribed differentially expressed genes. We found that eight cDNA clones in CAb.D5 and six cDNA clones in LN4.D6 cells were differentially expressed (Table 1).

Invasion of the extracellular matrix by tumour cells is an important step in the metastatic cascade (Ahmad & Hart 1997). Extracellular matrix is composed of basement membrane and interstitial connective tissue and type IV collagen is one of the important components of basement membrane. Collagen fibres such as those of types I, II, and III are main components of interstitial connective tissue. The enzymes produced by cancer cells, stromal cells and host leucocytes can degrade the extracellular matrix (Liotta *et al.* 1983). Because collagens are important components of the extracellular matrix which functions as a barrier for tumour cell invasion, the decrease in expression levels of collagens may facilitate cells to become metastatic. In addition, Martinelle-Catusse *et al.* (2001) demonstrated that alpha 3 collagen IV inhibits the invasive properties of an epithelial bronchial cell line, BZR. Moreover, a high level of type IV collagen expression and lower cell proliferation rate was observed in benign tumours compared with T4 thyroid carcinoma (Kusunoki *et al.* 2002). Therefore, in our study, expression of alpha 2 collagen IV and pro-alpha 1 collagen III in poorly metastatic CAb.D5 cells, but not in the highly metastatic LN4.D6, may be one of the important factors for CAb.D5 cells being less able to metastasize than LN4.D6 cells.

The clone FC-7, that corresponds to ribosomal protein L4, was differentially expressed in CAb.D5 cells. Previous studies have shown that expression levels of protein synthesis regulators change with metastasis. For example, the expression of ribosomal protein P2 increased in liver metastasis when compared with primary colon carcinoma and normal colonic mucosae (Elvin *et al.* 1988). However, the relative abundance of ribosomal phosphoprotein 2 was significantly higher in human breast fibroadenomas than in highly metastatic human adenocarcinomas (Sharp *et al.* 1990). In addition, Adams *et al.* (1992) reported that two genes encoding translation associated proteins, elongation factor 1-α and ubiquitin carboxyl-extension, were expressed at higher level in fibroadenomas than in carcinomas. Moreover, Daigneault *et al.* (1995) found that ribosomal protein L38, ribosomal protein S4, and acidic ribosomal phosphoprotein P1 were differentially expressed in non-metastatic SMF-Da rat rhabdomyosarcoma compared with metastatic SMF-Ai cell line. In our study, the differential expression of ribosomal protein L4 in poorly metastatic CAb.D5 cells, but not in highly metastatic LN4.D6, is consistent with the results of these groups. Taken together, these studies indicate that translation-associated genes were expressed at higher levels in non- or low-metastatic cell lines than in highly malignant or metastatic cells.

Cytokeratins are members of the intermediate filament multigene family and are classified in two major groups: type I (acidic cytokeratins: CK9-CK20) and type II cytokeratins (neutral or basic: CK1-CK8). The type and specificity of cytokeratins show significant differences of distribution among epithelial tissues and the expression pattern of cytokeratins varies with the cell state such as proliferation, differentiation, transformation and development (Sun *et al.* 1984; Simonishi *et al.* 2000). In addition, cytokeratins may be used as biomarkers for discriminating primary from metastatic carcinoma (Tot 2002). In this present study, we found that cytokeratin 8 (CK8) was expressed in CAb.D5, but not in the highly metastatic LN4.D6 cells. Similar to our finding, Zoli *et al.* (1997) reported that CK8 was poorly expressed, or not expressed at all, in two new cell lines derived from metastatic human breast carcinoma. In addition, Hansson *et al.* (2001) examined cytokeratin expression in normal, immortalized and malignant organotypic epithelial cells. They found that, although CK8 expression was relatively high (30–70%) in immortalized cells, no CK8 expression was detected in malignant epithelia.

Possible correlation between CK8 expression and inhibition of extracellular signal-related kinase (ERK) was shown in the metastatic human breast cancer cell line MDA-MB 231 (Seddighzadeh *et al.* 2000). Because there are biochemical and genetic implications concerning aberrant signalling in the ERK/mitogen-activated protein (MAP) kinase pathway contributing significantly to the transformed phenotype (Seddighzadeh *et al.* 2000), CK8 expression and ERK inhibition may be the most significant mechanisms for low metastatic potential of CAb.D5 cells in our study. Induction of CK8 expression by an ERK inhibitor in LN4.D6 cells and testing of metastatic ability will test this hypothesis.

Bocker *et al.* (2002) distinguished types of breast cancer by their cytokeratin expression patterns. Two differentiated cell phenotypes, glandular (CK5–/CK8/CK18/CK19 positive) and myoepithelial [smooth muscle actin (SMA)] exist within human mammary gland epithelium. More than 95% of breast cancer cases were of the pure glandular phenotype. However, carcinomas that are CK5+/CK8/CK18/CK19-negative and SMA-negative, and malignancies of myoepithelial type are exceptionally rare. Based on their study, it is probable that the CAb.D5 cell line would have a glandular phenotype and LN_4.D6 would have a myoepithelial phenotype (CAb.D5 expresses CK8; LN_4.D6 expresses CK5). In addition, differential expression of CK8 in CAb.D5 cells may be responsible for the low metastatic potential of this cell line. Future transfection studies of the *CK8* gene into LN4.D6 or human metastatic cell lines will elucidate whether CK8 has a different effect on tumour metastasis in different species. Furthermore, Al-Hajj *et al.* (2003) distinguished tumourigenic from non-tumourigenic breast cancer cells based on expression of cell-surface differentiation markers. Identification of cell-surface differentiation antigens as well as stem-cell differentiation markers (Bocker *et al.* 2002) will provide additional information for phenotypic characterization of CAb.D5 and LN_4.D6 cells in order to explain their metastatic potential.

Unlike the studies described here, expression of CK8 and tumour progression have been reported by other workers within benign and malignant breast tissue (Brotherick *et al.* 1998), medullary carcinoma of breast (Tot 2000), non-small cell lung cancer cell lines (Fujita *et al.* 1999 and Fukunaga *et al.* 2002) and colorectal cancer cells (Sasaki *et al.* 1998). In addition, Hembrough *et al.* (1997) has shown that CK8 promoted plasminogen activation which is involved in cellular migration and tumour invasion. Results from these studies indicate that type of cytokeratin expression changes from tissue to tissue and alteration in the expression of individual cytokeratins requires further investigation to determine any correlation with metastasis.

A further differentially expressed gene (FC-1), in forward-subtracted clones was aldehyde dehydrogenase. Expression of this gene was undetectable in LN4.D6 cells, however, it was

expressed at similar levels in CAb.D5, Mat.LyLu, AT1 and Rat.2 cell lines (Table 2). The metastatic relevance of this gene in rat mammary adenocarcinoma needs further investigation.

The clone FF-10 showed a significant homology (95%) with osteoblast/osteocyte factor 45 (OF45). Petersen *et al.* 2000 identified OF45 as a novel bone-specific cDNA in a rat bone marrow-derived osteoblasts using subtractive hybridization. These workers detected a single 2-kb mRNA transcript in bone and expression of its gene was not detected in other tissues. In contrast, the molecular weight of the FF-10 transcript was 1.3 kb in our work. Similar to their results, multitissue northern blot (Clontech) analysis showed that FF-10 was not expressed in other rat tissues, such as heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis in this study (data not shown). Specific expression of FF-10 transcript only in CAb.D5 cells, but not in other cells (Fig. 4), could indicate a possible function of this gene in low metastatic potential of CAb.D5 cells.

In reverse subtraction, five differentially expressed clones (RB-8, RB-9, RD-4, RE-1, RF-5) showed homology with known sequences (Table 1). A novel human mosaic protein LGN was first cloned and identified as protein that interacts with α-subunit of the heterodimeric GTP-binding protein, G_{i2} (Mochizuki *et al.* 1996). Full-length cDNA was obtained from the HeLa cell cDNA library and the appropriate protein contains 10 Leu-Gly-Asn repeats (LGN). mRNA of LGN was present in all human tissues. Similar to their results, LGN (RB-9 clone) was expressed in every cell examined in this present study. The expression level was 1.8-fold higher in LN4.D6 than it was in CAb.D5 (Table 2) and, as LGN interacts with GTP binding protein which mediates transmembrane signalling for receptors, it will be of specific interest for further investigation of metastatic relevance.

The clones RE-1 (CK5) and RF-5 (mouse epidermal type 1 cytokeratin) were expressed at the same frequency in LN4.D6 cells (Table 1), whereas no expression was detected in the CAb.D5 cell line. Similar to our result, Hansson *et al.* (2001) reported that CK5 expression was highest (30–70%) in malignant organotypic oral epithelia when compared with immortalized epithelia (less than 5%). Pencil *et al.* (1993) showed that CK7 expression was twofold higher in highly metastatic versus low metastatic cell line of rat 13762NF mammary adenocarcinoma cells. In addition, mouse epidermal cytokeratin (intermediate filament subunit II) was differentially expressed in placental tissue which invades the maternal uterus and shares features with invasion in malignancy (Kataoka *et al.* 2001). Again, these studies show possible involvement of cytokeratins in metastasis, but the type and function of a specific cytokeratin changes between tumour types.

BLAST search for partial cDNA sequences of clones FG-6 in forward subtraction and RE-12 in reverse subtraction showed no homology with known genes. Full-length cDNA sequence and metastatic relevance of these novel genes in other cell lines will be identified in further studies.

Finally, we examined the expression pattern of differentially expressed genes in two pairs of cancer cell lines: rat mammary adenocarcinoma (LN4.D6, CAb.D5) and rat prostate adenocarcinoma (MAT.LyLu and AT1). We found that the expression levels of all differentially expressed genes did not correlate with the metastatic potential of the cells. However, the expression level of FA-8 (alpha 2 collagen IV) showed a slight correlation with metastatic potential in which FA-8 was expressed in CAb.D5 only, but not in LN4.D6, and the expression level of FA-8 was found to be 1.5-fold higher in non-metastatic AT1 than in highly metastatic MAT.LyLu. Although there is no direct evidence from the literature concerning involvement of these differentially expressed genes in the metastatic process, it is likely that products of these genes may be directly or indirectly involved in metastasis, rather than simply being up-regulated as a result of altered cell phenotype. Further investigation of the expression profile of differentially expressed genes in a larger series of metastatic and non-metastatic mammary adenocarcinoma cell lines may explain this possibility.

In addition, in comparison with other cell lines, FF-10 was expressed only in CAb.D5, whereas RB-8, RE-1 and RF-5 were expressed only in LN4.D6 cells. Therefore, cell-specific expression of these differentially expressed genes will be the first target for future experiments related to metastatic potential of the cells.

In conclusion**,** we performed subtractive hybridization to differentiate genes expressed in highly metastatic LN4.D6 and poorly metastatic CAb.D5 cell lines. Eight cDNA clones in CAb.D5 and six cDNA clones in LN4.D6 were found to be differentially expressed. One cDNA clone in each cell line (FG-6 in CAb.D5; RE-12 in LN4.D6) has been shown to be novel. Identification of full-length cDNA sequences of these clones is under investigation. The expression profiles of differentially expressed genes in two pairs of rat adenocarcinoma cell lines did not show any correlation between expression of these genes and metastatic potential of the cell lines. However, it will be necessary to look at the expression pattern of these genes in a series of metastatic and non-metastatic cell lines from various mammary adenocarcinomas. Subsequently, further studies involving transfection of cDNA constructs of differentially expressed genes will help to elucidate the role of the genes in the metastatic process.

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REFERENCES

Adams SM, Helps NR, Sharp MGF (1992) Isolation and characterization of a novel gene with differential expression in benign and malignant human breast tumors. *Human Mol. Genet.* **1**, 91.

Ahmad A, Hart IR (1997) Mechanism of metastasis. *Crit. Rev. Oncol. Hematol.* **26**, 163.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) From the cover: prospective identification of tumorigenic breast cancer cells. *Proc. Natl Acad. Sci. USA* **100**, 3983.

Brotherick I, Robson CN, Browell DA, Shenfine J, White MD, Cunliffe WJ, Shenton BK, Egan M, Webb LA, Lunt LG, Young JR, Higgs MJ (1998) Cytokeratin expression in breast cancer: phenotypic changes associated with disease progression. *Cytometry* **32**, 301.

Bocker W, Moll R, Poremba C, Holland R, Van diest PJ, Dervan P, Burger H, Wai D, Ina Diello R, Brandt B, Herbst H, Schnidt A, Lerch MM, Buchwallow IB (2002) Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. *Lab. Invest.* **82**, 737.

Buckly ND, Carlsen SA (1988) Involvement of soybean agglutinin binding cells in the lymphatic metastasis of the R3230AC rat mammary adenocarcinoma. *Cancer Res.* **48**, 1451.

Carlsen SA, Barry M, Newton K (1990) The identification of a neural glycoshingolipid antigenic marker for metastatic cells in the R3230AC rat mammary adenocarcinoma. *Clin. Exp Metastasis* **8**, 141.

Carlsen SA, Xie Y, Whitfield DM, Pang H, Krepinsky JJ (1993) Isoglobotetraosy-ceramide is a marker for highly metastatic cells in rat mammary adenocarcinomas. *Cancer Res.* **53**, 2906.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acidguanidinium thyocynate-phenol-chloroform extraction. *Anal Biochem.* **162**, 156.

Daigneault L, Beaulieu R, Filion M, Gaboury L (1995) Cloning and identification of genes differentially expressed in metastatic and non-metastatic rat rhabdosarcoma cell lines. *Clin. Exp Metastasis* **13**, 345.

Dumonceaux T, Carlsen SA (2001) Isogloboside biosynthesis in metastatic R3230AC cells results from a decreased GM3 synthase activity. *Arch. Biochem. Biophys.* **389**, 187.

Elvin P, Kerr IB, McArdle CS, Birnie GD (1988) Isolation and preliminary characterization of cDNA clones representing mRNA associated with tumor progression and metastasis in colorectal cancer. *Br. J. Cancer* **57**, 36.

Fidler IJ, Nicolson GL (1987) The process of cancer invasion, metastasis. *Cancer Bull.* **39** 126.

Fujita J, Dobashi N, Ohtsuki Y, Ueda Y, Bandoh S, Yamadori I, Takahara J (1999) Detection of large molecular weight cytokeratin 8 as carrier protein of ca19-9 in non-small-cell lung cancer cell lines. *Br. J. Cancer* **81**, 769.

Fukunaga Y, Bandoh S, Fujita J, Yang Y, Ueda Y, Hojo S, Dohmoto K, Tojo Y, Takahara J, Ishida T (2002) Expression of cytokeratin 8 in lung cancer cell lines and measurement of serum cytokeratin 8 in lung cancer patients. *Lung Cancer* **38**, 31.

Hansson A, Bloor BK, Haigy Morgan PR, Ekstrand J, Grafstrom RC (2001) Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. *Oral Oncol*. **37**, 419.

Hembrough TA, Kralovich KR, Li L, Gonias SL (1997) Cytokeratin 8 released by breast carcinoma cells *in vitro* binds plasminogen and tissue-type plasminogen activator and promotes plasminogen activation. *Biochemical J*. **317**, 763.

Kataoka K, Nakajima A, Takata Y, Saito S, Huh N (2001) Screening for genes involved in tissue invasion based on placenta formation and cancer cell lines with low and high metastatic potential. *Cancer Lett*. **163**, 213.

Kusunoki T, Nishida S, Kimoto-Kinoshita S, Murata K, Satou T, Tomura T (2002). Type IV collagen, type IV collagenase activity and ability of cell proliferation in human thyroid tumours. *Asian J Surg*. **25**, 304.

Liotta LA, Rao CN, Barsky SH (1983) Tumor invasion and the extracellular matrix. *Lab. Invest*. **49**, 636.

Liotta LA, Steeg PS, Stetler-Stevenson WG (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* **64**, 327.

Martinelle-Catusse C, Polette M, Noel A, Gilles C, Dehan P, Munaut C, Colige A, Volders L, Monboisse JC, Foidart JM, Birembaut P (2001) Down-regulation of MT1-MMP expression by the alpha 3 chain of type IV collagen inhibits bronchial tumor cell line invasion. *Lab. Invest*. **81**, 167.

Mochizuki N, Cho G, Wen B, Insel PA (1996) Identification of cDNA cloning of a novel human mosaic protein, LGN, based on interaction with $G_{\alpha i2}$. *Gene* **181**, 39.

Pencil SD, Toh Y, Nicolson GL (1993) Candidate metastasis-associated genes of rat13762NF mammary adenocarcinoma. *Breast Cancer Res. Treatment* **25**, 165.

Petersen DN, Tkalcevic GT, Mansolf AM, Rivera-Gonzales R, Brown TA (2000) Identification of osteoblast/osteocyte factor 45 (OF45) a bone specific cDNA encoding anRGD-containing protein that is highly expressed in osteoblast and osteocytes. *J Biol. Chem*. **275**, 36172.

Sasaki M, Watanabe H, Jass JR, Ajioka Y, Kobayashi M, Hatakeyama K (1998) Immunoperoxidase staining for cytokeratin 8 and 18 is very sensitive for detection of occult node metastasis of colorectal cancer: a comparison with genetic analysis of K-ras. *Histopathology* **32**, 199.

Sharp MGF, Adams SM, Elvin P (1990) A sequence previously identified as metastasis related-encodes an acidic ribosomal phosphoprotein, P2. *Br. J. Cancer* **61**, 83.

Shimonishi T, Miyazaki K, Nakanuma Y (2000) Cytokeratin profile relates to histological subtypes and intrahepatic location of intrahepatic cholangiocarcinoma and primary sites of metastatic adenocarcinoma of liver. *Histopathology* **37**, 55.

Seddighzadeh M, Linder S, Shoshan MC, Auer G, Alaiya AA (2000) Inhibition of extracellular signal-related kinase $^1/_2$ activity of breast cacer cell line MDA-MB-231 leads to major alterations in the pattern of protein expression. *Electrophoresis* **21**, 2737.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucl. Acids. Res*. **23**, 1087–1088.

Sun T-T, Eichner R, Schermer A, Cooper D, Nelson WG, Weiss RA (1984) Classification expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In: Levine AJ, Vande Woude GF, Topp WC, Watson JD, eds. *The Transformed Phenotype: Cancer Cells*, p. 169. New York: Cold Spring Harbor Laboratory Press.

Tot T. (2000) The cytokeratin profile of medullary carcinoma of the breast. *Histopathology* **37**, 175.

Tot T (2002) Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarsinoma. *Eur J. Can Res*. **38**, 758.

Vedoy CG, Bengston MH, Sogayar MC (1999) Hunting for differentially expressed genes. *Brazilian J. Med. Biol. Res*. **32**, 877.

Wan JS, Erlander MG (1997) Cloning differentially expressed genes by using differential display and subtractive hybridization. *Meth. Mol. Biol*. **85**, 45.

Wang Z, Brown DD (1991) A gene expression screen. *Proc. Natl Acad. Sci. USA* **88**, 11505.

Yokota J (2000) Tumor progression and metastasis. *Carcinogenesis* **21**, 497.

Zoli W, Roncuzzi L, Zini N, Lenzi L, Gruppioni Barzanti F, Sensi A, Amadori D, Gasperi-Campani A (1997) Establishment and characterization of two new cell lines derived from human metastatic breast carcinomas. *Breast Cancer Res. Treatment* **43**, 141.