PERGAMON

European Journal of Cancer 37 (2001) 1554–1561

European Journal of Cancer

www.ejconline.com

Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line

A. Gemma[a,*], K. Takenaka[a], Y. Hosoya[a], K. Matuda[a], M. Seike[a], F. Kurimoto[a], Y. Ono[a], K. Uematsu[a], Y. Takeda[b], S. Hibino[a], A. Yoshimura[a], M. Shibuya[a], S. Kudoh[a]

[a]*Fourth Department of Internal Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan*
[b]*Deparment of Pulmonology, International Medical Center of Japan, 1-21-1, Toyama, Shinjyuku-ku, Tokyo 162-8655, Japan*

Received 17 November 2000; received in revised form 19 March 2001; accepted 18 April 2001

Abstract

Non-small cell lung cancer is associated with approximately 85% mortality due to its high metastatic potential. Therapeutic efforts have failed to produce a significant improvement in prognosis. In this situation, a better understanding of the key factors of metastasis may be useful for designing new molecular targets of therapy. In order to identify these factors, we compared the expression profiles of two subpopulations of an adenocarcinoma cell line with a high metastatic potential, PC9/f9 and PC9/f14, with the parent cell line, PC9, using a cDNA array. The expression of 15 genes was found to be significantly enhanced or reduced in the highly metastatic subpopulations. The expression of matrix metalloproteinase-2 (*MMP-2*), plasminogen activator inhibitor-1 (*PAI-1*) and interleukin-1 (*IL-1α*) were upregulated in the highly metastatic subpopulations, while the expression of carcinoembryonic antigen (*CEA*), *caspase-5*, *Fas* ligand, *Prk/FNK*, *cyclin E*, *cyclin B1*, *Ki-67*, proliferating cell nuclear antigen (*PCNA*), *Smad4*, macrophage proinflammatory human chemokine-3α (*MIP-3α*)/*LARC*, *Met* and *CD44* were downregulated. Data from the literature suggest that the altered expression of *MMP-2*, *PAI-1*, *IL-1α*, *CEA*, *caspase-5*, *Fas* ligand, *Prk/FNK* and *Smad4* promotes the highly metastatic phenotype. The differential expression of these genes was confirmed by Northern blot analysis, standard reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR. This analysis in subpopulations of a lung cancer cell line indicated that the highly metastatic potential of lung cancer may be induced not by an alteration in the expression of a single gene, but by the accumulation of alterations in the expression of several genes involved in extracellular matrix (ECM) adhesion disruption, ECM degradation, escape from apoptosis, and resistance to transforming growth factor-ß$_1$ (TGF-ß$_1$). Strategies for inhibiting metastasis of pulmonary adenocarcinoma should be designed accordingly.

Keywords: Lung cancer; Metastasis; cDNA array; Expression profile; Northern blot; RT-PCR

1. Introduction

Lung cancer is a major cause of death in Japan and Western countries, and a gradual increase in its incidence has been recognised. Approximately 70% of lung cancer patients will die from metastatic disease, even after complete resection of the primary tumour. Although chemotherapy modalities are widely used for inoperative lung cancer at present, their effect on survival remains far from satisfactory [1]. In this situation, inhibition of metastasis of lung cancer is thought to be one of the important therapeutic strategies. The molecules involved in the process of metastasis may be candidates for new molecular targets in the therapy of lung cancer.

Many proteins are involved in metastasis. Matrix metalloproteinases (MMPs) form a family of zinc-dependent matrix-degrading enzymes secreted by mesenchymal cells and tumour cells, and have been implicated in invasive cell behaviour [2,3]. Tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 and TIMP-2, have an abrogating effect on metastasis [4,5]. Urokinase-type plasminogen activator (uPA) plays a

* Corresponding author. Tel.: +81-3-3822-2132; fax: +81-3-5685-3075.
E-mail address: akihiko_gemmamed4@nms.ac.jp (A. Gemma).

0959-8049/01/$ - see front matter
PII: S0959-8049(01)00154-X

key role in tumour-associated proteolysis resulting in the invasion and dissemination of tumour cells [6]. uPA-catalysed proteolysis involves the urokinase-type plasminogen activator receptor (uPAR) [7]. The activity of the receptor-bound uPA is regulated by inhibitors of plasminogen activator, PAI-1 and PAI-2 [6]. Integrins comprise a supergene family of transmembrane proteins composed of noncovalently-associated α and ß subunits [8]. Integrins are involved in tumour cell adhesion, spreading and migration, thereby playing important roles in tumour cell growth and metastasis [9]. The proteins involved in cell growth and apoptosis also influence the process of metastasis [10,11]. These molecules involved in metastasis participate in one of four main activities: extracellular matrix (ECM) adhesion disruption, ECM degradation, cell cycle deregulation and escape from apoptosis.

In our previous study, we established a highly metastatic human lung adenocarcinoma cell line (PC9/f9) in an experimental metastasis model by repeated inoculation of PC9 cells in nude mice and subsequent culture of the tumour cells that were harvested from pulmonary metastatic foci [12]. PC9/f14 was established by five additional inoculations. The PC9/f9 cells [12] and PC9/f14 cells (data not shown) showed higher invasive activity in the matrigel invasion assay than the parent PC9 cells. In cell adhesion assays, PC9/f9 and PC9/f14 cells adhered to laminin, collagen type IV and fibronectin more strongly than PC9 cells [12].

It is of interest to evaluate the various molecules involved in metastasis. One approach to the identification of metastasis-associated genes would be to identify genes whose expression differs in the highly metastatic cell lines compared with the parent cell line they are derived from. The gene expression profiles of many genes can now be examined through cDNA and oligonucleotide microarrays, which are recently developed technologies. Thus, in this study, we analysed the expression profiles of the PC9, PC9/f9 and PC9/f14 lines using a cDNA array, and confirmed the results of the cDNA array analysis by northern blot, reverse transcription-polymerase chain reaction (RT-PCR) analyses and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Cell lines

The human lung adenocarcinoma cell line, PC9, and its highly metastatic sublines, PC9/f9 and PC9/f14, were used in this study. The PC9 line was established at the Tokyo Medical College, Tokyo, Japan. The PC9/f9 [12] and PC9/f14 lines were established at the Nippon Medical School. Briefly, a suspension of viable tumour cells of the PC9 line (5×10^5/mouse) was injected into the tail vein of nude mice. Male BALB/c nude mice were obtained from Charles River Co. (Yokohama, Japan) and were kept in a laminar air-flow cabinet under specific pathogen-free conditions. Six-week-old mice were used. Twelve weeks after injection, the mice were sacrificed and the lungs were examined. When pulmonary metastatic foci were seen, they were removed from the lung and minced in Roswell Park Memorial Institute (RPMI) 1640 medium with 20% fetal calf serum (FCS). After being grown and maintained for 4 weeks, this cell line was injected into other 6-week-old mice. The above process was performed nine times and resulted in the establishment of a highly metastatic cell line designated PC9/f9. Starting with the PC9/f9 line, the process was performed five times using SCID mice (Charles River Co., Yokohama, Japan) to yield the PC9/f14 line. To confirm the human origin of the xenografted tumour, *Alu* PCR was carried out [13]. In an experimental metastasis experiment, injection of PC9/f9 or PC9/f14 cells into the tail vein resulted in significantly more pulmonary metastases than injection of PC9 cells [12].

2.2. RNA isolation and cDNA array hybridisation

RNAs were prepared from the PC9, PC9/f9 and PC9/f14 cells using the standard protocols described previously in Ref. [14]. The mRNAs were isolated by incubation with oligo-dT-magnetic beads (Toyobo Co., Osaka, Japan), and the non-specific bindings to the beads were washed off. The GeneNavigator cDNA Array System — Cancer Selected — (Toyobo Co., Osaka, Japan) was used. One hundred seventy-seven species of human DNA fragments are duplicately spotted on the filter. The genes on the filter include cancer-related genes, housekeeping genes and non-mammalian genes as negative controls. A list of a set of genes on the filter is shown in web site: (http://www.toyobo.co.jp/seihin/xr/product/genenavi/genenavigator.html). To construct the probes, reverse transcription was performed using the RT, ReverTraAce (Toyobo Co., Osaka, Japan), random 9mer (Toyobo Co., Osaka, Japan) as the primer, and 5 μg of polyA RNA. The probes were labelled with biotin by incorporation of biotin-16-deoxyuracil triphosphate (dUTP) during the synthesis of cDNA. The filters were pre-incubated in 20 ml of PerfectHyb (Toyobo Co., Osaka, Japan) at 68°C for 30 min. The biotin-labelled probes were denatured and added to the pre-hybridisation solution. The filters were incubated in the hybridization mixture at 68°C overnight. After washing, specific signals on the filters were detected by the Imaging High Chemifluorescence Detection kit (Toyobo Co., Osaka, Japan). Vistra ECF substrate (AttoPhos) (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as the chemifluorescence substrate. A chemifluorescence image of the filter was

Table 1
List of primer sequences used in the polymerase chain reaction (PCR) amplification of the indicated genes for use as northern blot probes and in reverse transcriptase (RT)-PCR

Genes	Sense primer sequence	Antisense primer sequence
MMP-2	ggaagcatcaaatccgactgg	gtgaaaggagaagagcctgaagtg
PAI-1	ttgtggtctgtgtcaccgtatctc	gggaaagttctgtcctggtaggtc
IL-1α	tcaagatgaaggcaaagcacg	cctggctatgggataagcacaac
CEA	cctgtggtgggtaaatggtcagag	tgtgtgtgttgctgcggtatcc
FAS ligand	gtttatgagccagacaaatggagg	gaagtgaagatgctgccagtgg
Caspase-5	gcgtggctcatcaaatgtttacc	gcggtcctctctctttttttattgg
Prk/FNK	ttaggacccaagccctgaag	gctcacatcccaataaataagtgtc
Smad4	aaggtgaaggtgatgtttgggtc	gatgctctgtcttgggtaatccg
GAPDH	acagtccatgccatcactgcc	gcctgcttcaccaccttcttg

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CEA, carcinoembryonic antigen; MMP-2, matrix metalloproteinase-2; IL-1α, interleukin-1α; PAI-1, plasminogen activator inhibitor-1.

acquired by FluorImager (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.3. Analysis of the hybridisation signals

The gene expression images were quantified by measuring the intensity of the signals using Imagene (BioDiscovery, Los Angeles, CA, USA). The signal intensity among filters was compared by E-Gene Navigator Analysis (GeneticLab, Sapporo, Japan). The background threshold was set at the level of 3-fold of that of the negative control.

2.4. Northern blotting and standard RT-PCR

Northern blot analysis was performed as previously described in Ref. [15]. RT-PCR was performed to amplify each gene analysed in this study and the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as a control using the primers listed in Table 1. The amplified cDNA fragments were labelled with [^{32}P] deoxycyctodine triphosphate (dCTP) using a Prime-It RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA). These fragments, which differ from the cDNAs spotted on the filter for the cDNA array, were used as the probes in northern blot analysis. The northern blot filters were hybridised with a labelled probe, washed and exposed to X-ray film at −80°C overnight.

The Gene Amp XL RNA PCR kit (Perkin-Elmer, Roche, Branchburg, NJ, USA) was used for RT-PCR. RNase-free DNase-treated total RNA (250 ng) was used in the reaction as recommended by the manufacturer. PCR amplification (35 cycles) was performed using specific primers for the genes (Table 1).

2.5. Real-time quantitative RT-PCR

Quantitative PCR was performed using the Sequence Detector, ABI PRISM 7700, (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). The PCR primers and the TaqMan fluorogenic probes were designed using the Primer Express software program (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). Their sequences are shown in Table 2. One μg of each total RNA was reverse transcribed using a random hexamer and ReverTra Ace-α-(Toyobo Co., Osaka, Japan). A portion of the cDNA was used for quantitative PCR in a 50 μl volume using the designed primers, Taqman probes, and Master Mix, which was composed of PCR buffer, $MgCl_2$, deoxyadenosine triphosphate (dATP), dCTP, deoxyguanine triphosphate (dGTP), dUTP, AmpErase UNG and AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). The initial thermal cycle conditions were 50°C for 2 min and 95°C for 10 min, as recommended by the manufacturer. The cycle conditions were 95°C for 15 s and and 60°C for 1 min. The level of RNA of a gene was expressed as the ratio of the level of RNA of that gene to the level of *GAPDH* RNA in that sample.

Table 2
List of primer and probe sequences used in the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of the indicated genes

Gene	Sense primer sequence	Antisense primer sequence	Probe sequence
MMP-2	ccccaagctcatcgcagat	ggtccacgacggcatcc	cctggaatgccatccccgataacc
PAI-1	aaatcagacggcagcactgtct	cgtcaaggtcctacagcatcattac	actatactgagttcaccacgcccgatgg
IL-1α	cagttgcccatccaaacttgt	atagagggtggcccccc	acaaagcaagactactgggtgtgcttggc
CEA	ctgaggcaggagaatcgctt	gtggtgcgatctgggctc	aacccgggaggtggagattgcag
Fas ligand	cagatctactgggtggacagca	gcacagaggttggacaggga	tgggcccctccaggcacagtt
Caspase-5	gggctacactgtggttgacga	cagcaaatgccctcagca	aagaatctcactgccagggatatggagtca
Prk/FNK	cagagccgcgtcgcc	ggtgcagctcaatctcatttagg	agccgcatcagcgcgagaaga
Smad4	aaaacggccatcttcagcac	aggccagtaatgtccggga	acccgcctatgccgcccc
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	caagcttcccgttctcagcc

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CEA, carcinoembryonic antigen; MMP-2, matrix metalloproteinase-2; IL-1α, interleukin-1α; PAI-1, plasminogen activator inhibitor-1.

3. Results

3.1. Expression profiles of highly metastatic cell lines of human lung adenocarcinoma

PC9, PC9/f9, and PC9/f14 RNAs were prepared using the standard protocols as previously described in Ref. [14]. To avoid any influences from the cell culture conditions, we separately cultured each cell line in six bottles. Then, we performed cDNA array analysis, Northern blot hybridisation, and RT-PCR analysis of these three cell lines to identify the genes associated with metastasis. The results of hybridisation to the cDNA arrays on the filters provided by TOYOBO are shown in Fig. 1. The controls, including the *GAPDH* and *β-actin* genes, are located at the outer line in the opposite angle and are in duplicate. The standard curve was obtained by calculating serially diluted spots of *GAPDH* (Fig. 1(d)). The expression intensity of each gene was calculated by comparing it to the internal standard. We identified potential genes with a high metastatic activity by comparing the results of the cDNA array analysis of the

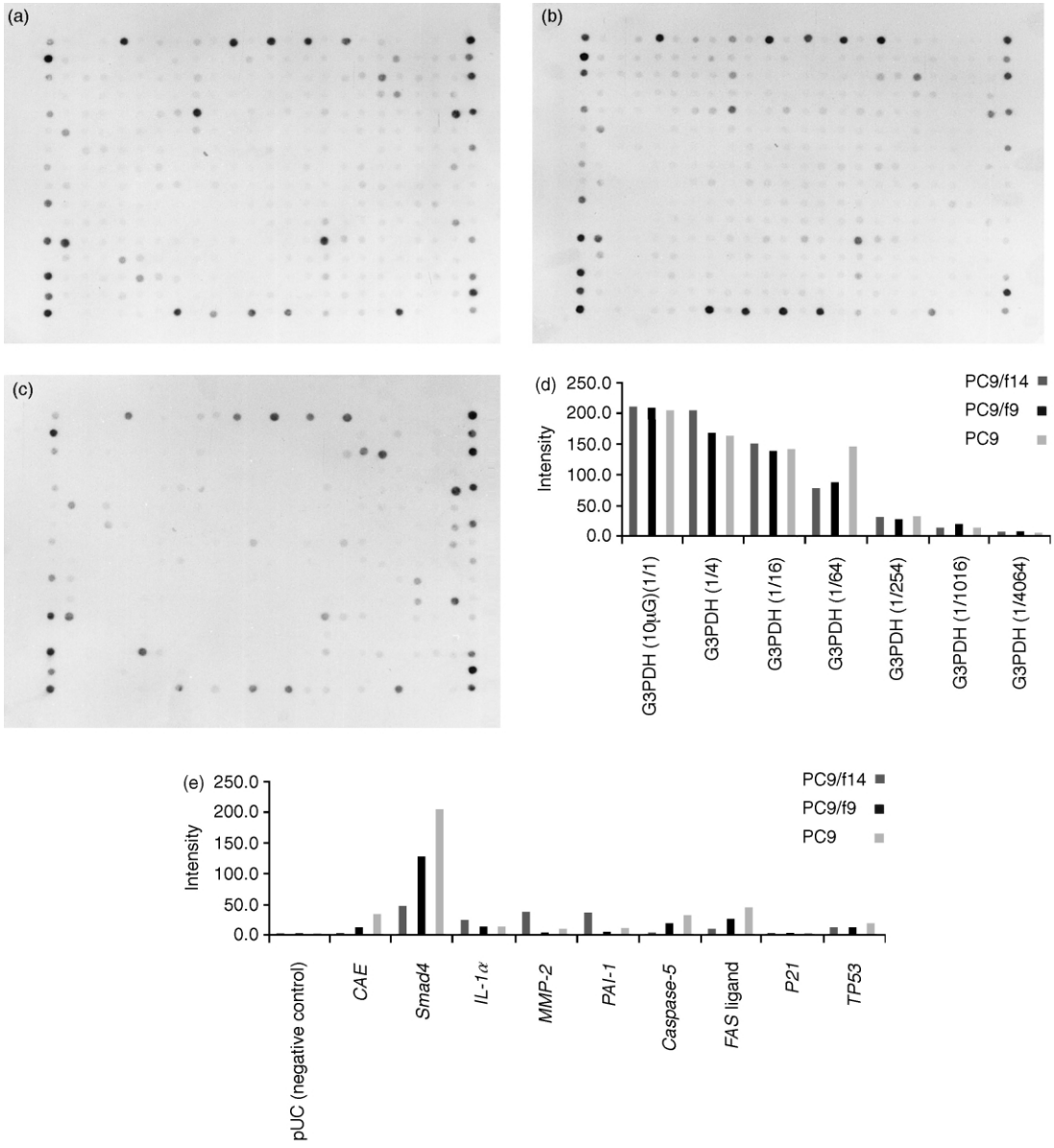

Fig. 1. cDNA array analysis of the expression of cancer-related genes in the PC9, PC9/f9 and PC9/f14 cell lines: (a) PC9 cell line; (b) PC9/f9 cell line; (c) PC9/f14 cell line; (d) Standard curves by calculated serially diluted spots of GAPDH; (e) Gene expression of pUC (negative control), *CEA*, *Smad4*, *IL-1α*, *MMP-2*, *PAI-1*, *Caspase-5*, *Fas* ligand, *p21* and *TP53* in PC9, PC9/f9 and PC9/f14. *IL-1α* *MMP-2* and *PAI-1* expression was increased and *CEA*, *Smad4*, *Caspase-5*, and *Fas* ligand expression was decreased in the highly metastatic subpopulations. *p21* and *TP53* did not change among the cell subpopulations (at least less than 3-fold). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CEA, carcinoembryonic antigen; MMP-2, matrix metalloproteinase-2; IL-1α, interleukin-1α; PAI-1, plasminogen activator inhibitor-1.

Table 3
Genes whose expression was upregulated in PC9/f9 or PC9/f14 on cDNA array analysis

Gene	PC9	PC9/f9	PC9/f14	Classes
MMP-2	7.2	3.6	43.2	Signalling intermediates
PAI-1	7.0	3.2	41.6	Signalling intermediates
IL-1α	7.0	12.4	27.2	Growth factor and hormones

MMP-2, matrix metalloproteinases-2; PAI-1, plasminogen activator inhibitor; IL-1α, interleukin-1α.

PC9, PC9/f9 and PC9/f14 cell lines. Some genes were upregulated and other genes were downregulated in the highly metastatic cell lines in comparison with those in the PC9 parent adenocarcinoma line (Fig. 1(e), Tables 3 and 4). The intensity of hybridisation on the filters of the cDNA array analysis were compared using Imagene software (BioDiscovery, Los Angeles, CA, USA). Table 3 shows the genes whose expression was upregulated in one or both of the highly metastatic cell lines. The expression of *MMP-2*, *PAI-1* and interleukin-1α (*IL-1α*) were upregulated in one of the highly metastatic cell lines, PC/f14. In addition, we found that the expression of several genes was suppressed in the highly metastatic cell lines. The expression of carcinoembryonic antigen (*CEA*), *caspase-5*, *Fas* ligand, *Prk/FNK*, *cyclin E*, *cyclin B1*, *Ki-67*, proliferating cell nuclear antigen (*PCNA*), *Smad4*, *LARC*, *Met* and *CD44* were downregulated in the PC9/f9 and/or PC9/f14 lines (Table 4).

Fig. 2 shows representative results of the northern blot analysis and the results of the RT-PCR analysis of the genes that were found to be differentially expressed on the cDNA array analysis. We could confirm the differential expression of *MMP-2*, *PAI-1*, *IL-1α*, *CEA*, *caspase-5*, Fas ligand, *Smad4* and *Prk/FNK* between the highly metastatic cell lines and the parent cell line, by northern blot, RT-PCR and/or real-time RT-PCR analyses (Figs. 2 and 3, Table 5). RT-PCR and real-time quantitative RT-PCR were each performed two times for each gene.

Table 5
Differential expression of genes between the PC9/f9 or PC9/f14 cell lines and the parent PC9 cell line using real-time RT-PCR

Gene	PC9	PC9/f9	PC9/f14
MMP-2	1.00	3.47	157.07
PAI-1	1.00	0.24	183.40
IL-1α	1.00	2.23	18.26
CEA	1.00	0.10	0.03
Fas ligand	1.00	0.45	0.01
Caspase-5	1.00	0.19	0.03
Prk/FNK	1.00	0.02	0.02
Smad4	1.00	0.02	0.00

CEA, carcinoembryonic antigen; MMP-2, matrix metalloproteinase-2; IL-1α, interleukin-1α; PAI-1, plasminogen activator inhibitor-1.

4. Discussion

The highly metastatic cell lines, PC9/f9 [12] and PC9/f14, showed a higher invasive activity in the matrigel invasion assay than the PC9 cells. In cell adhesion assays, PC9/f9 [12] and PC9/f14 cells (data not shown) adhered to laminin, collagen type IV and fibronectin more strongly than PC9 cells. Pretreatment of PC9/f9 cells with anti-ß1 monoclonal antibodies suppressed lung metastasis by more than 50% in animal studies [12]. FACS analysis showed that the integrins, α4ß1 and α5ß1, were expressed on PC9/f9 cells, but not on the parent PC9 cells [12]. PC9/f9 cells adhered to collagen type IV via α2ß1, and to fibronectin mainly via α5ß1 and moderately via α4ß1 [12]. These data suggest that altered expression of ß1 integrins leads to the increased metastatic potential of PC9/f9 cells in comparison with the parent PC9 cells.

In the present study, we compared the expression profiles of the highly metastatic cell lines, PC9/f9 and

Table 4
Genes whose expression was downregulated in PC9/f9 or PC9/f14 on cDNA array analysis

Gene	PC9	PC9/f9	PC9/f14	Classes
CEA	30.3	8.7	0.8	Tumour suppressor genes/apoptosis
FAS ligand	37.7	24.0	5.9	Apoptosis
Caspase-5	20.9	19.3	0.5	Apoptosis
Prk/FNK	18.0	2.9	2.3	Signalling intermediates
Cyclin E	69.9	20.8	5.5	Cell cycle protein
Ki-67	44.5	8.3	18.8	Cell cycle protein
Cyclin B1	41.2	23.2	5.7	Cell cycle protein
PCNA	28.6	6.4	8.0	Cell cycle protein
Smad4	205.9	141.4	57.9	Regulatory transcription factors
MIP-3α/LARC	23.1	15.4	1.0	Growth factor and hormones
Met	18.3	24.6	2.7	Membrane receptor
CD44(HCAM)	32.7	39.3	6.4	Cell adhesion proteons

PCNA, proliferating cell nuclear antigen; MIP-3α, macrophage proinflammatory human chemokine-3α; CEA, carcinoembryonic antigen.

PC9/f14, with that of the parent cell line, PC9, using a cDNA array. *MMP-2*, *PAI-1* and *IL-1α* expression were upregulated in one of the highly metastatic lines, PC9/f14. MMPs comprise a family of metal-dependent endopeptidases secreted by mesenchymal and tumour cells. MMP-2, MMP-3 and MMP-9 play a major role in cancer invasion and metastasis [16,17]. TIMPs which are also expressed by cancer cells and mesenchymal cells, abrogate the invasive properties of tumour cells [4]. The correlation between tumour secretion of MMP-2 and MMP-9, and experimental metastasis was based on the measurement of type-IV collagen and gelatin degradation [16]. These enzymatic activities as well as inhibitor activity are present in tumour-conditioned media [18]. In the present study, the PC9/f14 line expressed a higher level of MMP-2 than the PC9 and PC9/f9 lines, although the three cell lines expressed similar levels of TIMPs and other MMPs. uPA, uPAR, PAI-1, and PAI-2 may be involved in cancer metastasis [6]. uPA converts inactive plasminogen to active plasmin, which degrades the extracellular matrix protein directly or indirectly during the tumour invasion process [6]. PAI-1 seems to act in synergy with u-PA to promote the invasion process, and is associated with the aggressiveness of lung tumours [19]. PAI-1 expression was also significantly upregulated in the PC9/f14 line. IL-1α was shown to enhance the adhesion of A549 lung carcinoma and M6 melanoma cells to the vascular surface both *in vitro* and *in vivo*, suggesting that IL-1 proteins facilitate the metastatic process [20]. The PC9/f14 cell line expressed a higher level of IL-1α than the parent PC9 cell line.

Genes whose expression was downregulated in the PC9/f9 and/or PC9/f14 cell lines included those encoding *CEA*, *caspase-5*, *Fas* ligand, *Prk/FNK cyclin E*, *cyclin B1*, *Ki-67*, *PCNA*, *Smad4*, *LARC*, *MIP-3α*, *Met* and *CD44*. CEA is a highly glycosylated cell surface glycoprotein belonging to the immunoglobulin superfamily, and is involved in adhesion mechanisms. Transfection of the *CEA* gene in human rhabdomyosarcoma cells was reported not to affect the subcutaneous growth of rhabdomyosarcoma in nude mice, but it strongly inhibited the tumour's metastatic ability to the lungs and adrenals after intravenous (i.v.) injection [21]. Impairment of the metastatic potential is correlated with a reduction in the homotypic adhesion properties of the cells [21]. The function of *caspase-5* is unknown. Proteases of the caspase family are involved in both programmed cell death, and immune and inflammatory responses. The *caspase-5* gene was reported to be mutated in tumours with microsatellite instability [22]. One advantage of cells with a mutated caspase-5 gene may be inhibition of apoptosis or inhibition of the inflammatory response [22]. Fas and Fas ligand are complementary receptor-ligand proteins that induce apoptosis [23,24]. Fas ligand expression is normally restricted to the spleen, lung, intestine, uterus, testis and eye [23,24]. Both the incidence and number of metastasis were reported to be increased in Fas ligand-deficient mice [10]. The Fas–Fas ligand interaction can suppress

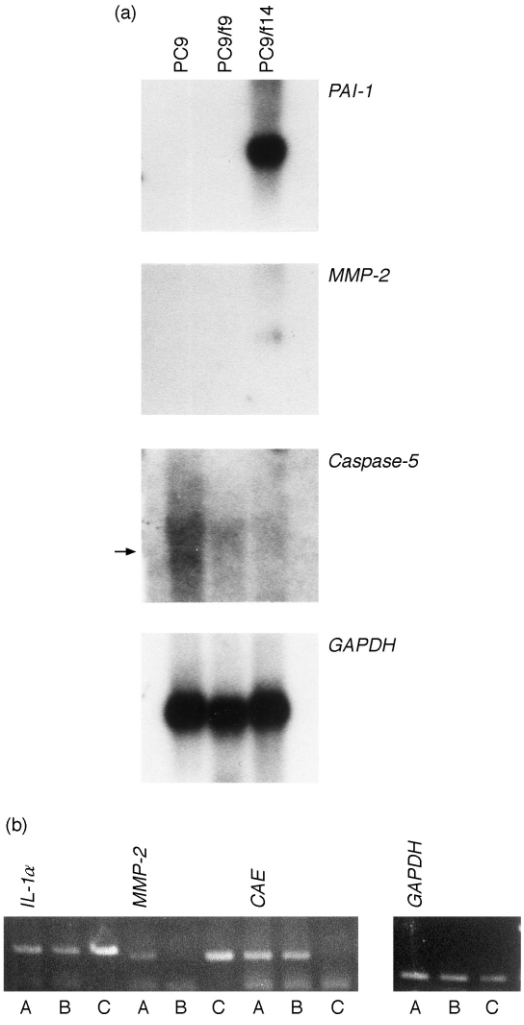

Fig. 2. Results of northern blot analysis and RT-PCR of genes that were differentially expressed in the PC9, PC9/f9 and PC9/f14 cell lines: (a) northern blot analysis of *PAI-1*, *MMP-2*, *caspase-5* and *GAPDH*; (b) RT-PCR of *IL-1α*, *MMP-2*, *CEA* and *GAPDH*. A: PC9 cell line; B: PC9/f9 cell line; C: PC9/f14 cell line.

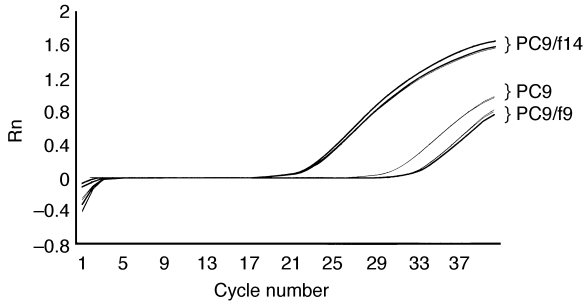

Fig. 3. Results of real-time quantitative RT-PCR of *PAI-1* expression in the PC9, PC9/f9 and PC9/f14 cell lines. Rn, normalised reporter (signal intensity of reporter/signal intensity of passive reference).

metastasis, and its dysfunction may be linked to metastatic progression [10]. Actually, tumours without Fas and Fas ligand expression had a significantly higher incidence of metastasis in many clinical studies [25–27]. Prk/FNK has been cloned and characterised as a putative serine/threonine kinase, and shows strong homology to mouse fnk and polo family kinases [28]. *Prk/FNK* expression was reported to be downregulated in primary lung tumour samples [28]. Fnk regulates the early signalling events in the cell cycle progression stimulated by growth factors [29]. *CEA* and *Prk/FNK* expression were reduced in the PC9/f9 and PC9/f14 lines. *Caspase-5* and *Fas* ligand which are involved in apoptosis, were weakly expressed in the PC9/f14 line.

Cyclin E [30], cyclin B1 [31], Ki-67 [32], PCNA [33] and Smad4 [34] are involved in cell cycle regulation and tumour growth. The *in vitro* growth rates of the cell lines, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay and [^{3}H]-thymidine incorporation assay, showed that PC9/f9 cells have a similar ability of growth *in vitro* relative to PC9 cells [12], while PC9/f14 cells have a higher growth ability than PC9 cells (data not shown). The reduced expression of *cyclin E*, *cyclinB1*, *Ki-67* and *PCNA* in the highly metastatic cell lines in this study indicates that these cell cycle regulators may not be involved in the highly metastatic ability of the cell lines. The altered expression of these cell cycle regulators seems to be a secondary change, when comparing the expressions of these genes in the cell lines to the growth ability of the cell lines. In contrast, the level of *Smad4* expression changed in parallel with cell growth ability. *Smad4* is a member of the signalling pathway of the transforming growth factor-ß (TGF-β) superfamily [34]. TGF-$ß_1$ regulates the proliferation of normal epithelial cells, and resistance to TGF-$ß_1$ growth inhibition is a common feature of human cancers [35]. The growth advantage of the PC9/f14 cell line may be due to resistance to TGF-$ß_1$. MIP-3α/LARC expression has been described in macrophages, eosinophils, dendritic cells, colon cancer, lung cancer, pancreatic cancer, leukaemia, lymphoma and melanoma [36,37]. It stimulates the growth or migration of pancreatic cancer [38]. Met-hepatocyte growth factor/scatter factor (HGF/SF) signalling was suggested to enhance tumour growth and metastasis of ras-transformed NIH3T3 cells [39]. Overexpression of CD44 was reported to promote lung colonisation during micrometastasis of murine fibrosarcoma cells [40]. These molecules might be regulated by a negative- feedback mechanism in highly metastatic cell lines.

In summary, although *in vivo* and *in vitro* selection cannot be ruled out as contributing to the metastatic phenotype, the upregulation of *MMP-2*, *PAI-1* and *IL-1α* expression and downregulation of *CEA*, *caspase-5*, *Fas* ligand, *Prk/FNK* and *Smad4* expression seem to be involved in the highly metastatic phenotype of the PC9/f14 and/or PC9/f9 cell lines. During the repeated incubation and culture of the PC9 parent cell line, a reduction of *CEA* and *Prk/FNK* expression occurred in the PC9/f9 cells. Upon further repeated incubation and culture, the expression of *MMP-2*, *PAI-1* and *IL-1α* increased and the expression of *caspase-5*, *Fas* ligand and *Smad4* decreased. The results of the present study indicate that the highly metastatic phenotype of the PC9/f9 and PC9/f14 cell lines resulted not from a single gene alteration, but from an accumulation of alterations in the expression of various genes that play specific roles.

Acknowledgements

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

References

1. Meta-analysis Group. *Chemotherapy for Non-Small Cell Lung Cancer*. Non-small Cell Lung Cancer Collaborative Group. Cochrane Database Syst Rev 2000, CD002139. MRC Clinical Trials Unit, Cancer Division, 5 Shaftesbury Road, Cambridge, UK.
2. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu Rev Cell Biol* 1993, **9**, 541–573.
3. Kahari VM, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med* 1999, **31**, 34–45.
4. Schultz RM, Silberman S, Persky B, *et al*. Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. *Cancer Res* 1988, **48**, 5539–5545.
5. Ahonen M, Baker AH, Kahari VM. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer Res* 1998, **58**, 2310–2315.
6. Dano K, Behrendt N, Brunner N, *et al*. The urokinase receptor. Protein structure and role in plasminogen activation and cancer invasion. *Fibrinolysis* 1994, **8**(Suppl. 1), 189–203.
7. Pepper MS, Sappino AP, Stocklin R, *et al*. Upregulation of urokinase receptor expression on migrating endothelial cells. *J Cell Biol* 1993, **122**, 673–684.
8. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992, **69**, 11–25.
9. Liotta LA. Tumor invasion and metastases — role of the extracellular matrix: Rhoads memorial award lecture. *Cancer Res* 1986, **46**, 1–7.
10. Owen-Schaub LB, van Golen KL, Hill LL, *et al*. Fas and Fas ligand interactions suppress melanoma lung metastasis. *J Exp Med* 1998, **188**, 1717–1723.
11. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* 1995, **1**, 149–153.
12. Kiyoshi Takenaka, Masahiko Shibuya, Yuichiro Takeda, *et al*. Altered expression and function of ß1 integrins in a highly metastatic human adenocarcinoma cell line. *Int J Oncol* 2001, in press.
13. Tagle DA, Collins FC. An optimized *Alu*-PCR primer pair for human-specific amplification of YACs and somatic cell hybrids. *Hum Mol Genet* 1992, **1**, 121–122.

14. Jiang W, Zhang YJ, Kahn SM, *et al*. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc Natl Acad Sci USA* 1993, **90**, 9026–9030.
15. Gemma A, Takenoshita S, Hagiwara K, *et al*. Molecular analysis of the cyclin-dependent kinase inhibitor genes p15INK4B/MTS2, p16 INK4/MTS1, p18 and p19 in human cancer cell lines. *Int J Cancer* 1996, **68**, 605–611.
16. Bernhard EJ, Muschel RJ, Hughes EN. Mr 92,000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells. *Cancer Res* 1990, **50**, 3872–3877.
17. Liotta LA, Stetler-Stevenson WG. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res* 1991, **51**, 5054s–5059s.
18. Lefebvre V, Vaes G. The enzymatic evaluation of procollagenase and collagenase inhibitors in crude biological media. *Biochim Biophys Acta* 1989, **992**, 355–361.
19. Robert C, Bolon I, Gazzeri S, *et al*. Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor. *Clin Cancer Res* 1999, **5**, 2094–2102.
20. Lauri D, Bertomeu MC, Orr FW, *et al*. Interleukin-1 increases tumor cell adhesion to endothelial cells through an RGD dependent mechanism: in vitro and in vivo studies. *Clin Exp Metastasis* 1990, **8**, 27–32.
21. Landuzzi L, Frabetti F, Rossi I, *et al*. Expression of transduced carcinoembryonic antigen gene in human rhabdomyosarcoma inhibits metastasis. *Cancer Res* 1996, **56**, 4503–4508.
22. Schwartz S Jr, Yamamoto H, Navarro M, *et al*. Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer Res* 1999, **59**, 2995–3002.
23. Suda T, Takahashi T, Golstein P, *et al*. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993, **75**, 1169–1178.
24. French LE, Hahne M, Viard I, *et al*. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J Cell Biol* 1996, **133**, 335–343.
25. Mann B, Gratchev A, Bohm C, *et al*. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. *Br J Cancer* 1999, **79**, 1262–1269.
26. Koomagi R, Volm M. Expression of Fas (CD95/APO-1) and Fas ligand in lung cancer, its prognostic and predictive relevance. *Int J Cancer* 1999, **84**, 239–243.
27. Shibakita M, Tachibana M, Dhar DK, *et al*. Prognostic significance of Fas and Fas ligand expressions in human esophageal cancer. *Clin Cancer Res* 1999, **5**, 2464–2469.
28. Li B, Ouyang B, Pan H, *et al*. Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. *J Biol Chem* 1996, **271**, 19402–19408.
29. Donohue PJ, Alberts GF, Guo Y, *et al*. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. *J Biol Chem* 1995, **270**, 10351–10357.
30. Koff A, Cross F, Fisher A, *et al*. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* 1991, **66**, 1217–1228.
31. Gautier J, Minshull J, Lohka M, *et al*. Cyclin is a component of maturationpromoting factor from Xenopus. *Cell* 1990, **60**, 487–494.
32. Malizia G, Trejdosiewicz LK, Wood GM, *et al*. The microenvironment of coeliac disease: T cell phenotypes and expression of the T2 'T blast' antigen by small bowel lymphocytes. *Clin Exp Immunol* 1985, **60**, 437–446.
33. Takasaki Y, Deng JS, Tan EM. A nuclear antigen associated with cell proliferation and blast transformation. *J Exp Med* 1981, **154**, 1899–1909.
34. Hahn SA, Schutte M, Hoque AT, *et al*. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996, **271**, 350–353.
35. Gemma A, Hagiwara K, Vincent F, *et al*. hSmad5 gene, a human hSmad family member: its full length cDNA, genomic structure, promoter region and mutation analysis in human tumors. *Oncogene* 1998, **16**, 951–956.
36. Rossi DL, Vicari AP, Franz-Bacon K, *et al*. Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3alpha and MIP-3beta. *J Immunol* 1997, **158**, 1033–1036.
37. Power CA, Church DJ, Meyer A, *et al*. Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3alpha from lung dendritic cells. *J Exp Med* 1997, **186**, 825–835.
38. Kleeff J, Kusama T, Rossi DL, *et al*. Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer. *Int J Cancer* 1999, **81**, 650–657.
39. Webb CP, Taylor GA, Jeffers M, *et al*. Evidence for a role of Met-HGF/SF during Ras-mediated tumorigenesis/metastasis. *Oncogene* 1998, **17**, 2019–2025.
40. Kogerman P, Sy MS, Culp LA. Overexpressed human CD44s promotes lung colonization during micrometastasis of murine fibrosarcoma cells: facilitated retention in the lung vasculature. *Proc Natl Acad Sci USA* 1997, **94**, 13233–13238.