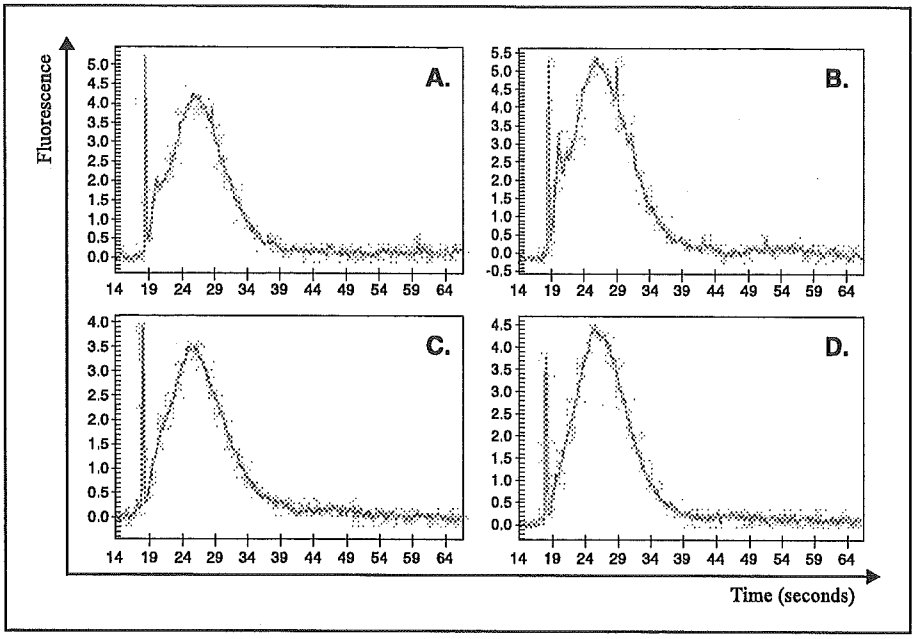

Figure 2. Representative electropherograms of successful RNA amplification from the normal epithelium (*A*), papilloma (*B*), dysplasia (*C*), and invasive carcinoma (*D*). They showed a hump peak and one marker peak. No contamination of the ribosomal RNA was detected.

genes, such as *Mmp2* (Rat matrix metalloproteinase 2 mRNA), *Ctsh* (Rat mRNA for cathepsin H), *Arhc* (Mouse rhoC mRNA), and *C1s* (Rat mRNA for serine protease) were also included.

Real-time RT-PCR Assay. In order to verify our cDNA microarray data, we did a real-time RT-PCR for some genes identified in Table 2*A* and *B*. The expression levels of real-time RT-PCR and the cDNA microarray are presented as the log ratio average of each three experiments (Fig. 6). These results corresponded very well to the microarray data for all six genes, thus strongly supporting the reliability and rationale of our strategy.

Discussion

To date, several studies have shown the gene expression patterns in human ESCC using DNA microarray technologies (7–13). Regarding animal models, the molecular mechanisms of carcinogenesis in the breast (14, 15), thyroid (16), liver (17), and stomach

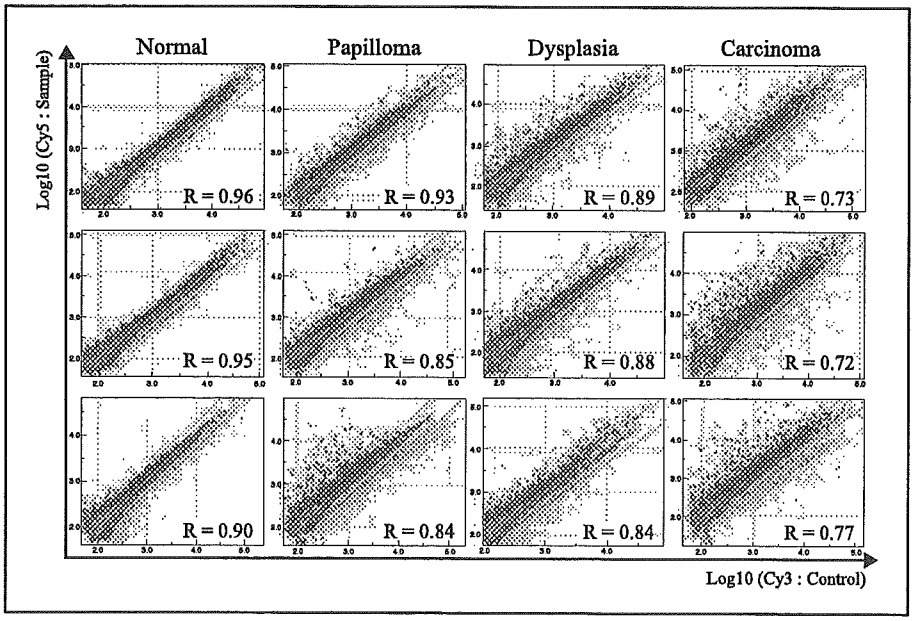

Figure 3. Intensity plots of a cDNA microarray in three samples each from normal epithelium, papilloma, dysplasia, and invasive carcinoma. *Red*, up-regulation into each sample; *green*, down-regulation; *blue*, unchanged based on the Rosetta Luminator error model ($P < 0.01$). The highest correlation coefficient ($R = 0.93 \pm 0.03$) was seen between the normal epithelium and control (mixture of three normal epithelium). The correlation coefficients in papilloma ($R = 0.87 \pm 0.05$) and dysplasia ($R = 0.87 \pm 0.03$) were intermediate. The gene expression profiles of invasive carcinoma and the control showed the lowest correlation coefficient ($R = 0.74 \pm 0.02$).

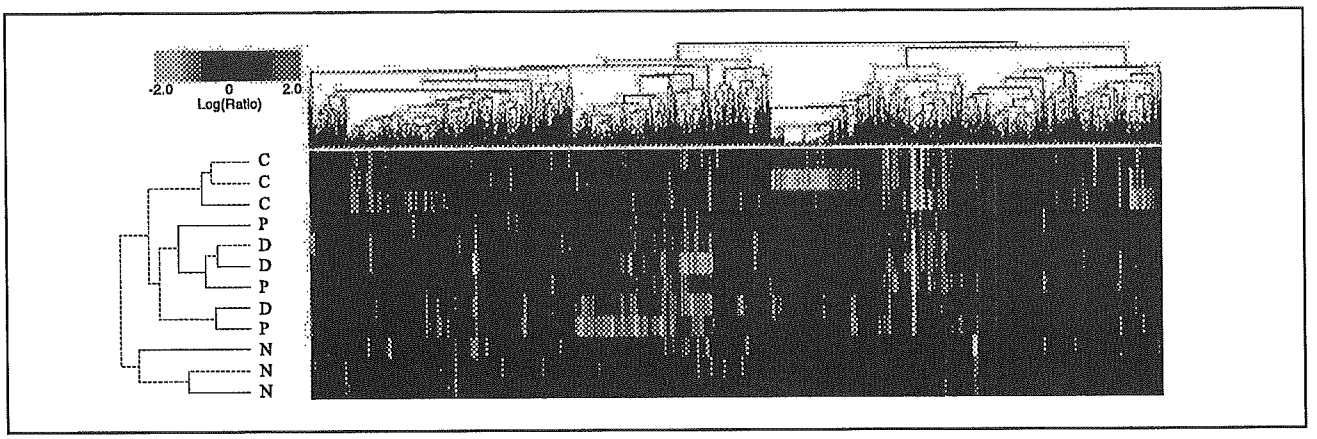

Figure 4. A hierarchical clustering analysis using 3,644 differentially expressed genes ($P < 0.01$). Three categories of normal epithelium, papilloma (dysplasia), and invasive carcinoma were well classified, whereas the expression patterns of papilloma and dysplasia were indistinguishable. *N*, normal epithelium; *P*, papilloma; *D*, dysplasia; *C*, invasive carcinoma.

(18) were evaluated using DNA microarrays, whereas no information is available for the gene expression profiles in an esophageal carcinogenesis model. The technical feasibility of a DNA microarray analysis for microdissected specimens in a rat liver carcinogenesis model has been recently reported (35). However, most of the previous studies examined the gene expression profiles in bulk tumor tissues, thus suggesting that these results might reflect heterogeneous expression profiles. In this study, we thus employed a technique of LMD to specifically isolate the cells from each esophageal lesion. The quality of RNA extracted from each specimen, which is extremely important for a DNA microarray analysis, was also carefully taken into account. We examined the quality of RNA using Agilent 2100 bioanalyzer, and we only used the specimens with high-quality RNA (Fig. 2). By combining the LMD and cDNA microarray, we showed that the number of differentially expressed genes markedly increased step-by-step from normal epithelium to dysplasia (papilloma) to invasive carcinoma (Fig. 3). It seems theoretically reasonable that the differences in the gene expression profiles between normal and dysplasia (papilloma) was smaller than those between normal and invasive carcinoma. Lu et al. (7) have investigated gene expression profiles of five different stages during initiation and progression of human ESCC. They observed that the constant number of differentially expressed genes between the normal stage and stages of mild dysplasia (492 genes), moderate dysplasia (481 genes), carcinoma *in situ* (473 genes), and squamous cell carcinoma (501 genes). One possible explanation for the reason why, unlike us, they did not find a step-by-step increase in the number of differentially expressed genes might be attributable to differences in the species examined. Alternatively, we could reasonably show such a stepwise increase (Fig. 3), owing to the application of LMD to purify the cells from each lesion.

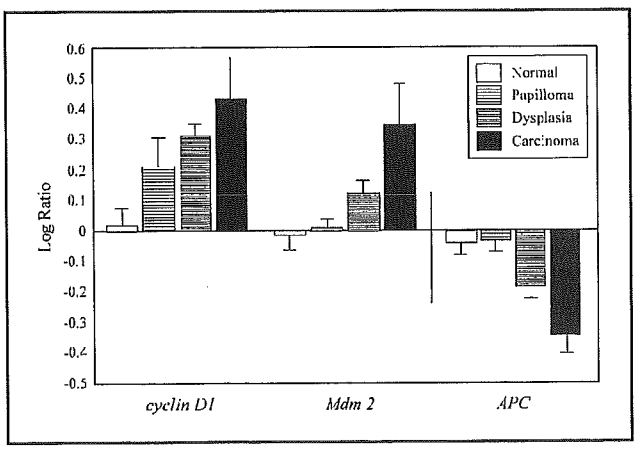

Figure 5. Results of a cDNA microarray for three genes, which are well known to be cancer-related genes. Expression levels of *cyclin D1* (Rat mRNA for *cyclin D1*) and *Mdm2* (Mouse *Mdm2* gene) were elevated in invasive carcinoma followed by dysplasia. Expression of *APC* (Mouse adenomatous polyposis coli) was markedly decreased in invasive carcinoma followed by dysplasia.

We observed the increased expression of *cyclin D1* not only in the dysplasia and carcinoma but also in the papilloma. A hierarchical clustering analysis did not distinguish the papilloma from dysplasia in our rat model. Furthermore, some previous reports have suggested that papilloma and hyperplastic lesions might be precancerous changes in rat esophageal carcinoma, as deduced from sequential morphologic studies (19, 36). On the other hand, human esophageal papilloma is a rare entity, which is not generally associated with the development of ESCC (37), whereas dysplasia has been well established as a precancerous lesion. These findings suggest that the association of rat papilloma with esophageal carcinogenesis may be different from that of human papilloma. The detailed differences remain to be determined.

The careful purification of cells from the normal epithelium, dysplasia, and invasive carcinoma, subsequent RNA isolation, and a cDNA microarray analysis identified 50 genes whose expression was associated with the development of rat ESCC (Table 2). For this step in the statistical analysis, we adopted the Fisher criterion because it takes variance into account unlike the Euclidean distance or the criterion based on a fold change. The up-regulated elements included the genes associated with cell adhesion (*Tmem8, Colla1, Lamc2,* and *Coll2a1*) and cell growth (*LynB* and *Arhc*). The genes coding for proteases (*Mmp2, Ctsh,* and *C1s*) were also up-regulated. Kan et al. (13) showed that the gene expression profiles of cancer tissues and those of cancer cell lines to be considerably different in human esophageal cancers using a cDNA microarray. One of the clearest distinctions was that the expression of proteases such as

Table 2. Genes associated with esophageal carcinogenesis

Accession no.	Symbol	Description	Gene ontology	Log ratio (average)		
				Normal	Dysplasia	Carcinoma
A. Forty-one up-regulated genes associated with the esophageal carcinogenesis						
U65656	*Mmp2**	Rat gelatinase A mRNA, complete cds. (matrix metalloproteinase 2)	metallopeptidase activity, carcinogenesis	−0.141	0.287	0.976
AB012026	*Krt2- 6a*	Mouse gene for keratin 6α, exon 2.	structural constituent of cytoskeleton	−0.117	1.194	1.637
X68273	*Cd68*	Mouse mRNA for macrosialin.	CD68 antigen, integral to membrane	−0.098	0.399	0.888
U32115	*Gp38*	Rat E11 antigen epitope (OTS- 8) mRNA, complete cds.	lung development	0.022	0.311	0.739
AB045293	*Tmem8*	Mouse mRNA for M83 protein, complete cds.	cell adhesion molecule activity	−0.231	0.444	0.964
AF000302	*LynB*	Rat Lyn B tyrosine kinase (LynB) mRNA, complete cds.	cell growth and/or maintenance	−0.038	0.263	0.760
U14914	*Ptprr*	Rat protein tyrosine phosphatase PC12- PTP1 (PTP) mRNA, complete cds.	transmembrane receptor activity	−0.525	0.121	0.606
D63774	*Krt14*	Rat mRNA for keratin 14, partial cds.	structural constituent of cytoskeleton	−0.103	0.297	0.932
X58861	*C1qa*	Mouse mRNA for complement subcomponent C1Q α- chain.	complement activity	−0.268	0.401	0.996
Y00708	*Ctsh**	Rat mRNA for cathepsin H (EC 3.4.22.16).	cysteine- type peptidase activity, carcinogenesis	−0.094	0.299	0.865
X71899	*Plaur*	Rat uPAR- 2 mRNA for urinary plasminogen activator receptor 2.	Plasminogen activator, urokinase receptor	−0.209	0.018	0.528
X56602	*G1p2*	Mouse mRNA Interferon- induced 15- kDa protein.	immune response	−0.243	0.056	0.692
AF004811	*Msn*	Rat moesin mRNA, complete cds.	structural molecule activity	0.052	0.470	0.897
Z78279	*Col1a1*	Rat mRNA for collagen α1 type I.	cell adhesion molecule activity	−0.169	0.047	0.961
AF165887	*Bcat1*	Rat cytosolic branch chain aminotransferase BCATc mRNA, partial cds.	transferase activity	−0.102	0.122	0.893
X15963	*Cox5a*	Mouse mRNA for cytochrome *c* oxidase subunit Va.	oxidoreductase activity	−0.039	0.148	0.772
AF176840	*Chst5*	Mouse intestine *N*- acetylglucosamine 6- *O*- sulfotransferase (I- GlcNAc- 6- ST) mRNA, complete cds.	transferase activity	−0.152	0.726	1.321
U43327	*Lamc2*	Mouse laminin γ2 chain (B2t) mRNA, complete cds.	cell adhesion molecule activity	0.020	0.329	0.740
D88250	*C1s**	Rat mRNA for serine protease, complete cds.	serine protease, carcinogenesis	0.082	0.606	1.139
U07201	*Asns*	Rat asparagine synthetase mRNA, secondary transcript, complete cds.	ligase activity	0.092	0.591	1.016
AF083269	*Arpc1b*	Rat p41- Arc mRNA, complete cds.	structural constituent of cytoskeleton, cell motility	0.110	0.439	0.826
U57362	*Col12a1*	Rat collagen XII α1 (Col12a1) mRNA, partial cds.	cell adhesion molecule activity	−0.041	0.131	0.992
AF010405	*Foxq1*	Mouse fork head transcription factor (Hfh- 1L) gene, complete cds.	transcription factor activity	0.114	0.369	0.766
AF135059	*Fbn1*	Rat fibrillin- 1 mRNA, complete cds.	cell adhesion molecule activity, metanephrogenesis	−0.036	0.281	0.914

(Continued on the following page)

Table 2. Genes associated with esophageal carcinogenesis (Cont'd)

Accession no.	Symbol	Description	Gene ontology	Log ratio (average)		
				Normal	Dysplasia	Carcinoma
M83143	*Siat1*	Rat β- galactoside- alpha 2,6- sialyltransferase mRNA.	transferase activity	−0.119	0.535	1.107
M38135	*Ctsh*	Rat cathepsin H (RCHII) mRNA.	cysteine- type peptidase activity, carcinogenesis	−0.003	0.387	0.947
AAF35394	*Gga2*	γ-adaptin related protein, GGA2	protein transporter activity	0.170	0.367	0.780
AF159593	*Plscr1*	Mouse phospholipid scramblase 1 mRNA, complete cds.	calcium ion binding	−0.120	0.260	0.957
X80638	*Arhc*	Mouse rhoC mRNA.	cell growth and/or maintenance, carcinogenesis	−0.002	0.153	0.784
U81829	*Calu*	Mouse calumenin mRNA, complete cds.	calcium ion binding protein	−0.241	−0.034	0.339
Z18877	*Oas1*	Rat mRNA for 2'-5'- oligoadenylate synthetase.	2'- 5'- oligoadenylate synthetase activity	−0.169	0.082	0.647
X51615	*Cx26*	Rat RNA for connexin protein Cx26.	gap junction membrane channel protein	0.031	0.306	1.000
BAA24267	*Farp1*	CDEP	Rho guanyl- nucleotide exchange factor activity	−0.277	0.032	0.624
AJ400844	*Pilra*	Mouse mRNA for imunoglobulin-like cell surface receptor FDF03.	protein binding	−0.339	−0.010	0.528
D86041	*Ddah1*	Rat mRNA for *N*-G,*N*-G-dimethylarginine dimethylaminohydrolase	hydrolase activity	−0.113	0.020	0.694
X70369	*Col3a1*	Rat mRNA for pro α1 collagen type III.	extracellular matrix structural constituent	−0.102	0.261	1.000
L03294	*Lpl*	Rat lipoprotein lipase mRNA, complete cds.	lipid transporter activity	−0.145	0.232	0.825
U06755	*Cnn3*	Rat Sprague- Dawley acidic calponin mRNA, complete cds.	actin bundling activity, calmodulin binding	0.018	0.272	0.930
J03026	*Mgp*	Rat matrix Gla protein mRNA, complete cds.	calcium ion binding	−0.138	0.442	1.446
AB008571	*Fhl2**	Rat mRNA for DRAL, complete cds.	protein binding	−0.088	0.273	0.905
L00193	*Krt1- 10*	Mouse epidermal keratin type I intermediate filament gene, exons 2 to 8.	structural constituent of cytoskeleton	0.111	0.943	1.354
B. Nine down-regulated genes associated with the esophageal carcinogenesis						
AF001896	*Aldh1a1**	Rat aldehyde dehydrogenase mRNA, complete cds.	oxidoreductase activity	0.022	−0.432	−0.911
BAA87047	*Mtvr2*	C184M protein	receptor activity	0.105	−0.297	−0.733
AF151982	*Slpi*	Rat secretory leukocyte protease inhibitor mRNA, complete cds.	inflammatory response regulate	0.032	−0.532	−1.150
AF121081	*Slc37a2*	Mouse cAMP inducible 2 protein (Ci2) mRNA, complete cds.	glycerol- 3- phosphate transporter activity	−0.109	−0.322	−0.918
AJ132356	*ArsB*	Mouse ARS component B gene, exons 1- 3.	cytokine activity	−0.108	−0.759	−1.427
AF017393	*Cyp2f1**	Rat cytochrome *P*4502F4 (CYP4502F4) mRNA, complete cds.	monooxygenase activity	−0.165	−0.508	−1.248
U04842	*Egf*	Rat preproepidermal growth factor mRNA, complete cds.	growth factor activity	0.121	−0.335	−0.757
CAA69194	*Pir*	Pirin	transcription cofactor activity	0.144	−0.198	−0.612
AAC17966	*Dscam*	Down syndrome cell adhesion molecule	cell adhesion molecule activity	0.066	−0.136	−0.667

*The gene expression levels were validated by real-time RT-PCR, and results are shown in Fig. 6.

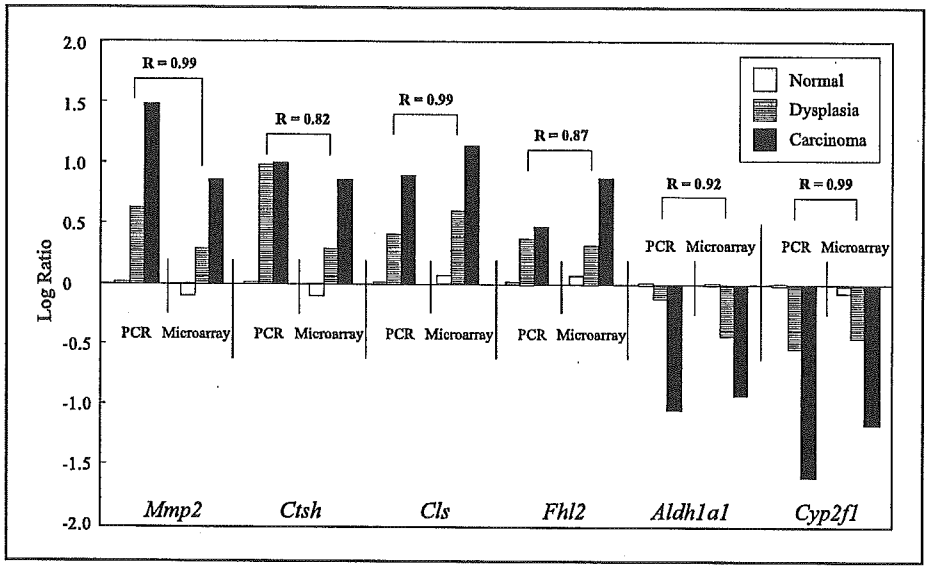

Figure 6. Validation of cDNA microarray data by real-time quantitative RT-PCR. Expression levels of the selected six genes using real-time RT-PCR corresponded very well to those using a cDNA microarray for the normal epithelium, dysplasia, and invasive carcinoma (R = 0.82-0.99). Results verify the reliability of our cDNA microarray experiments.

matrix metalloproteinases and plasminogen activators was high in most cancer tissues, whereas it was low in the cancer cell lines. In the present study, we found the stepwise increases of proteases including matrix metalloproteinase 2 (*Mmp2*), Cathepsin H (*Ctsh*), a serine protease (*C1s*) as shown in Fig. 6. The expression of *Mmp2* has been found to be correlated with cancer cell migration and invasion (38, 39). Recently, these proteases have gotten a lot of attention as a therapeutic target for invasive carcinomas (40). A lysosomal cysteine protease, *Ctsh* plays a central role in the cellular processes such as the degradation of intracellular proteins, extracellular matrix remodeling, and apoptosis (41). The overexpression of *Ctsh* has been associated with the malignant progression of a variety of cancers (42, 43). The family of enzymes, known as serine protease (*C1s*), supports many biological functions for cancer cells, including the activation of growth and angiogenic factors and the activation of other proteases for invasion and metastasis. Several genes encoding enzymes associated with cell adhesion and degradation, including urinary plasminogen activator receptor 2 (*Plaur*) also increased (Table 2*A*). In addition, a protease inhibitor (*Slpi*) showed stepwise a decrease from the normal epithelium to dysplasia to invasive carcinoma. These findings support the importance of examining the gene expression profiles of "*in vivo*" cancer cells isolated by a LMD and may also suggest the limitation of "*in vitro*" studies of cancer cell lines. This genome-wide global information obtained herein will hopefully contribute to an improved understanding of the molecular alterations that occur during the development of ESCC, and also potentially help to establish novel diagnostic and therapeutic targets. Because the present study proves the usefulness of this approach, other studies are presently under way to obtain additional information on these molecular pathways using esophageal specimens from patients with ESCC.

Acknowledgments

Received 8/9/2004; revised 10/16/2004; accepted 11/10/2004.

Grant support: Long-range Research Initiative project of the Japan Chemical Industry Association; Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research 15390398; Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant-in-Aid for Scientific Research on Priority Areas 15023245; and a Health and Labor Sciences Research Grant on Hepatitis and BSE 14230801, Ministry of Health, Labor, and Welfare of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Haruko Yasunami, Mayumi Oda, Mayumi Ikeda, and Kazue Ogata for their excellent technical assistance.

References

1. Matsufuji H, Ueo H, Mori M, Kuwano H, Sugimachi K. Enhancement of esophageal carcinogenesis induced in rats by *N*-amyl-*N*-methylnitrosamine in the presence of 12-*O*-tetradecanoylphorbol-13-acetate. J Natl Cancer Inst 1987;79:1123–9.
2. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–32.
3. Cho KR, Vogelstein B. Genetic alterations in the adenoma–carcinoma sequence. Cancer 1992;70: 1727–3.
4. Shinozaki H, Ozawa S, Ando N, et al. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. Clin Cancer Res 1996;2:1155–61.
5. Opitz OG, Harada H, Suliman Y, et al. A mouse model of human oral-esophageal cancer. J Clin Invest 2002;110:761–9.
6. Wang LD, Hong JY, Qiu SL, Gao H, Yang CS. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. Cancer Res 1993;53:1783–7.
7. Lu J, Liu Z, Xiong M, et al. Gene expression profile changes in initiation and progression of squamous cell carcinoma of esophagus. Int J Cancer 2001; 91:288–94.
8. Kihara C, Tsunoda T, Tanaka T, et al. Prediction of sensitivity of esophageal tumors to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. Cancer Res 2001;61:6474–9.
9. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992;256:668–70.
10. Luo A, Kong J, Hu G, et al. Discovery of Ca2+-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. Oncogene 2004;23:1291–9.
11. Hu YC, Lam KY, Law S, Wong J, Srivastava G. Profiling of differentially expressed cancer-related genes in esophageal squamous cell carcinoma (ESCC) using human cancer cDNA arrays: overexpression of oncogene MET correlates with tumor differentiation in ESCC. Clin Cancer Res 2001;7:3519–25.

12. Hu YC, Lam KY, Law S, Wong J, Srivastava G. Identification of differentially expressed genes in esophageal squamous cell carcinoma (ESCC) by cDNA expression array: overexpression of Fra-1, Neogenin, Id-1, and CDC25B genes in ESCC. Clin Cancer Res 2001;7:2213–21.
13. Kan T, Shimada Y, Sato F, et al. Gene expression profiling in human esophageal cancers using cDNA microarray. Biochem Biophys Res Commun 2001;286: 792–801.
14. Wang Y, Hu L, Yao R, et al. Altered gene expression profile in chemically induced rat mammary adenocarcinomas and its modulation by an aromatase inhibitor. Oncogene 2001;20:7710–21.
15. Kuramoto T, Morimura K, Yamashita S, et al. Etiology-specific gene expression profiles in rat mammary carcinomas. Cancer Res 2002;62:3592–7.
16. Ying H, Suzuki H, Furumoto H, et al. Alterations in genomic profiles during tumor progression in a mouse model of follicular thyroid carcinoma. Carcinogenesis 2003;24:1467–79.
17. Tellgren A, Wood TJ, Flores-Morales A, Torndal UB, Eriksson L, Norstedt G. Differentially expressed transcripts in neoplastic hepatic nodules and neonatal rat liver studied by cDNA microarray analysis. Int J Cancer 2003;104:131–8.
18. Abe M, Yamashita S, Kuramoto T, et al. Global expression analysis of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced rat stomach carcinomas using oligonucleotide microarrays. Carcinogenesis 2003;24:861–7.
19. Sasajima K, Taniguchi Y, Okazak S, et al. Sequential morphological studies of the esophageal carcinoma of rats induced by *N*-methyl-*N*-amylnitrosamine. Eur J Cancer Clin Oncol 1982;18:559–64.
20. Iizuka T, Ichimura S, Kawachi T. Esophageal carcinoma in rats induced by *N*-amyl-*N*-methylnitrosamine. Gann 1980;71:94–9.
21. Reuber MD. Histopathology of preneoplastic and neoplastic lesions of the esophagus in BUF rats ingesting diethylnitrosamine. J Natl Cancer Inst 1977;58:313–21.
22. Mori M, Mimori K, Yoshikawa Y, et al. Analysis of the gene-expression profile regarding the progression of human gastric carcinoma. Surgery 2002;131:S39–47.
23. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci U S A 1990;87:1663–7.
24. Eberwine J, Yeh H, Miyashiro K, et al. Analysis of gene expression in single live neurons. Proc Natl Acad Sci U S A 1992;89:3010–4.
25. Utsunomiya T, Okamoto M, Hashimoto M, et al. A gene-expression signature can quantify the degree of rat hepatic fibrosis. J Hepatol 2004;41:399–406.
26. Yang YH, Dudoit S, Luu P, et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30:e15.
27. Luo L, Salunga RC, Guo H, et al. Gene expression profiles of laser-captured adjacent neuronal subtypes. Nat Med 1999;5:117–22.
28. Okabe H, Satoh S, Kato T, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129–37.
29. Zhang T, Nanney LB, Luongo C, et al. Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients. Cancer Res 1997;57: 169–75.
30. Jenkins TD, Mueller A, Odze R, et al. Cyclin D1 overexpression combined with *N*-nitrosomethylbenzylamine increases dysplasia and cellular proliferation in murine esophageal squamous epithelium. Oncogene 1999;18:59–66.
31. Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. Proc Natl Acad Sci U S A 1998;95:15608–12.
32. Ganguli G, Abecassis J, Wasylyk B. MDM2 induces hyperplasia and premalignant lesions when expressed in the basal layer of the epidermis. EMBO J 2000; 19:5135–47.
33. Morin PJ, Vogelstein B, Kinzler KW. Apoptosis and APC in colorectal tumorigenesis. Proc Natl Acad Sci U S A 1996;93:7950–4.
34. Iizuka N, Oka M, Yamada-Okabe H, et al. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. Lancet 2003;361:923–9.
35. Michel C, Desdouets C, Sacre-Salem B, Gautier JC, Roberts R, Boitier E. Liver gene expression profiles of rats treated with clofibric acid: comparison of whole liver and laser capture microdissected liver. Am J Pathol 2003;163:2191–9.
36. Napalkov NP, Pozharisski KM. Morphogenesis of experimental tumors of the esophagus. J Natl Cancer Inst 1969;42:927–40.
37. Talamini G, Capelli P, Zamboni G, et al. Alcohol, smoking and papillomavirus infection as risk factors for esophageal squamous-cell papilloma and esophageal squamous-cell carcinoma in Italy. Int J Cancer 2000;86:874–8.
38. Davies B, Waxman J, Wasan H, et al. Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. Cancer Res 1993; 53:5365–9.
39. Mori M, Mimori K, Shiraishi T, et al. Analysis of MT1-MMP and MMP2 expression in human gastric cancers. Int J Cancer 1997;74:316–21.
40. Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. Oncogene 2003;22:8716–22.
41. Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 1997;59:63–88.
42. del Re EC, Shuja S, Cai J, Murnane MJ. Alterations in cathepsin H activity and protein patterns in human colorectal carcinomas. Br J Cancer 2000;82: 1317–26.
43. Waghray A, Keppler D, Sloane BF, Schuger L, Chen YQ. Analysis of a truncated form of cathepsin H in human prostate tumor cells. J Biol Chem 2002;277: 11533–8.

Clinicopathologic and Biological Significance of Kallikrein 6 Overexpression in Human Gastric Cancer

Hisashi Nagahara,[1,2] Koshi Mimori,[1] Tohru Utsunomiya,[1] Graham F. Barnard,[3] Masaichi Ohira,[2] Kosei Hirakawa,[2] and Masaki Mori[1]

Abstract **Purpose:** Human kallikrein genes (*KLK*) have been reported to be involved in human malignancies and several *KLK*s are promising biomarkers of prostate, ovarian, testicular, and breast cancers. Herein, we investigated the clinicopathologic and biological significance of *KLK6* gene expression in human gastric cancer.

Patients and Methods: Using real-time reverse transcription-PCR, we analyzed the *KLK6* expression status with respect to various clinicopathologic variables in 66 patients with gastric cancer. In addition, we established a *KLK6* stably suppressed gastric cancer cell line (MKN28) using small interfering RNA–mediated gene silencing, and investigated its effects on the cell proliferation rate, cell cycle, and invasiveness.

Results: The *KLK6* gene expression in cancerous tissue (0.37 ± 0.53) was significantly ($P < 0.000001$) higher than that in noncancerous tissue (0.026 ± 0.060). Elevated *KLK6* expression was significantly associated with lymphatic invasion ($P = 0.03$). Furthermore, patients with a high *KLK6* expression had a significantly poorer survival rate than those with a low *KLK6* expression ($P = 0.03$). Therefore, we showed that *KLK6* gene silencing with *KLK6* small interfering RNA effectively suppressed the cell proliferation rate ($P = 0.002$), cell population in the S phase ($P < 0.01$), and invasiveness ($P < 0.01$) in comparison to mock-transfected cells.

Conclusions: The *KLK6* gene is markedly overexpressed in gastric cancer tissue and its expression status may be a powerful prognostic indicator for patients with gastric cancer. Our findings also suggest that *KLK6* may possibly be a novel target for gastric cancer therapy by gene-silencing procedures.

The kallikrein gene family of secreted serine proteases, consisting of 15 genes, localizes tandemly on chromosome 19q13.4 and shows significant homologies at both the nucleotide and the protein levels (1–5). The human kallikrein gene 6 (*KLK6*), encoding human kallikrein 6 protein (hK6), has been cloned independently by three groups. Using a differential display PCR technique from breast cancer cell lines, Anisowicz et al. cloned the full-length cDNA of *KLK6*, named *protease M* (6). They showed that *protease M* was strongly expressed in breast cancer cell lines and in ovarian cancer tissues and cell lines. Little et al. cloned the identical cDNA, which they named *ZYME*, from brain tissue of a patient with Alzheimer's disease (7). Yamashiro et al. cloned the gene, which they called *NEUROSIN*, from a cDNA library prepared from a human colorectal cancer cell line (8).

Recent studies have suggested that human *KLK*s are involved in human carcinogenesis and that several *KLK*s are promising biomarkers of prostate, ovarian, testicular, and breast cancers (3, 5). For example, the *KLK3* gene encodes prostate-specific antigen (hK3), which is a currently available cancer-specific marker, and is widely used for the screening, diagnosis, and management of prostate cancer (9, 10). *KLK2* protein (hK2) can be another useful diagnostic marker for prostate cancer (11, 12). Many other *KLK*s have also been expected to act as tumor biomarkers (13–18). In addition, more recent evidence also implicates *KLK*s in many cancer-related processes, including cell-growth regulation, angiogenesis, invasion, and metastasis (4, 5). Regarding KLK6, several authors have reported that the *KLK6* mRNA was highly expressed in ovarian cancer tissue and that hK6 could be a useful serum biomarker for the diagnosis and monitoring of ovarian cancer (19, 20). However, no information is available on *KLK6* expression in human gastric cancer, the second most common cancer in Japan.

In the present study, we therefore examined the clinicopathologic and prognostic significance of *KLK6* expression

Authors' Affiliations: [1]Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu; [2]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan; [3]Department of Medicine, University of Massachusetts, Worcester, Massachusetts

Received 4/30/05; revised 7/5/05; accepted 7/12/05.

Grant support: Grant-in-aid for Scientific Research (B) (15390398, 15390379, 16390381); grant-in-aid for Scientific Research (C) (15591412, 15591411) from Japan Society for the Promotion of Science; grant-in-aid for Exploratory Research (16659337) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. Public Trust Haraguchi Memorial Cancer Research Fund and the Sankyo Foundation of Life Science also supported this study.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Masaki Mori, Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 81-874-0838, Japan. Phone: 81-977-27-1650; Fax: 81-977-27-1651; E-mail: mmori@beppu.kyushu-u.ac.jp.

doi:10.1158/1078-0432.CCR-05-0943

in gastric cancers. Furthermore, we investigated the association of such biological behaviors, as cell growth and invasiveness, with *KLK6* gene expression when suppressed by gene silencing with small interfering RNA (siRNA) in a gastric cancer cell line.

Materials and Methods

Cell lines and tissue samples. The cell lines derived from human gastric cancer, including AZ521, KATOIII, MKN1, MKN7, MKN28, MKN45, MKN74, NUGC3, NUGC4, and human fibroblast cell line, KMST6, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), and maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% humidified CO_2 atmosphere.

The 66 tumor samples and the matched control samples taken from normal tissue located far from the tumor site of gastric cancers were frozen in liquid nitrogen immediately after a surgical resection, and were kept at −90°C until RNA extraction. The surgical samples were obtained at the Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan. None of these patients received preoperative treatment, such as radiation or chemotherapy. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board.

Clinicopathologic data. All data, including sex, histology, serosal invasion, lymph node metastasis, lymphatic invasion, vascular invasion, and clinical stage were obtained from the clinical and pathologic records.

RNA preparation and reverse transcription. The total RNA was isolated by the modified acid guanidinium-phenol-chloroform procedure with DNase (21). cDNA was synthesized from 2.5 μg of total RNA as described previously (22).

Oligonucleotide primers for KLK6 gene amplification by PCR. The primer sequences for *KLK6* were: 5′-CATGGCGGACCCTGCGACAAGAC-3′ and 5′-TGGATCACAGCCCGGACAACAGAA-3′. The forward primer was located in exon 2, whereas the reverse primer was located in exon 3. The length of the amplicon was 215 bp. The amplification was done for 28 cycles of 1 minute at 95°C, 1 minute at 57°C, 1 minute at 72°C. An 8 μL aliquot of each reaction mixture was size-fractionated in a 2% agarose gel and visualized by ethidium bromide staining. To ensure that the RNA was not degraded, a PCR assay with primers specific for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was carried out in each case, except that only 22 cycles were done under the following cycling conditions: 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The primer sequences for the *GAPDH* amplification were: 5′-TTGGTATCGTGGAAGGACTCA-3′ and 5′-TGTCATCATATTTGGCAGGTT-3′ (23). The length of this amplicon was 249 bp.

Real-time quantitative reverse transcription-PCR. The real-time monitoring of the PCR reactions was done using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Tokyo, Japan). The amplification conditions of the 40 cycles consisted of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 10 seconds. The products were then subjected to a temperature gradient from 68°C to 95°C at 0.1°C/s with continuous fluorescence monitoring to produce a melting curve of the products. After proportional background adjustment, the fit point method was used 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. The standard curve was produced by measuring the crossing point of each standard value (2-fold serially diluted cDNAs of MKN28) and plotting them against the logarithmic value of the concentration. Concentrations of each sample were then calculated by setting their crossing points to the standard curve. The expression levels were normalized by the *GAPDH* mRNA expression (23). We classified the 66 cases into two groups using the mean expression level of *KLK6* mRNA in tumor tissue (0.37): i.e., a high-expression group (>0.37, $n = 20$) and a low-expression group (<0.37, $n = 46$).

Immunohistochemistry. Immunohistochemical studies of hK6 were done on surgical specimens from patients with gastric cancer using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissue specimens. All sections were counterstained with hematoxylin. The primary mouse monoclonal antibodies against hK6 (MCA2158, Serotec, Ltd., Oxfordshire, United Kingdom) were used at dilutions of 1:500.

All sections were independently examined by three investigators (H. Nagahara, K. Mimori, H. Inoue). We scored the expression as negative for hK6 when <10% of the carcinoma cells were stained in an examined area of a specimen. We examined hK6 protein expression in tumor and the corresponding normal tissues from 18 representative gastric cancer cases among 66 cases. In 18 selected cases, 6 cases showed a lower expression level of *KLK6* mRNA in gastric cancer tissues than in noncancerous tissues, whereas the remaining 12 cases exhibited a higher *KLK6* mRNA expression in tumor than normal tissues.

Small interfering RNA transfection. The expression vector, pSilencer 3.1-H1 hygro (Ambion Inc., Austin, TX) was used for the expression of siRNA. A hairpin siRNA designed to target the *KLK6* gene (5′-GATCCCGTAAGTTGGTGCATGGCGGATTCAAGAGATCCGCCATGCACCAACTTATTTTTTGGAAA-3′, sense strand) was inserted into the pSilencer according to the manufacturer's instructions, and then it was transfected into the gastric cancer cell line (MKN28) by the Lipofect-AMINE method (Life Technologies, Inc., Tokyo, Japan) as described previously (24). Two stably transfected clones were selected after hygromycin treatment (800 μg/mL) and used for the subsequent experiments. A mock vector-transfected clone of the cell line was used as a control.

Western blot analysis. Total protein was extracted from the samples with a radioimmunoprecipitation assay buffer. Aliquots of total protein were applied to 12% acrylamide gradient gels. After electrophoresis, the samples were electroblotted onto a polyvinylidene membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 Å for 50 minutes at 4°C. The hK6 protein was detected using mouse monoclonal antibody (Serotec) at dilutions of 1:1,000. The protein levels of hK6 were normalized to the level of β-actin protein (Cytoskeleton, Inc., Denver, CO) at dilutions of 1:1,000. The blots were developed with horseradish peroxidase–linked anti-mouse immunoglobulin (Promega, Inc., Madison, WI) at dilutions of 1:5,000. Signals were detected using Supersignal (Pierce, Inc., Rockford, IL).

In vitro proliferation assay. Cells were plated at a density of 5×10^4 cells per well in three 6-cm plates and were harvested and counted on days 3, 7, and 10. The medium was changed every 72 hours. This experiment was done in triplicate.

Cell cycle analysis. Cells (2.0×10^6) were preincubated for 48 hours in serum-free medium at 37°C and then were kept in medium with serum (10% FBS) for 18 hours at 37°C. The cells were harvested and fixed in 70% ethanol at −20°C. Next, the cells were washed and resuspended in propidium iodide staining buffer (5 μg/mL propidium iodide and 0.25 mg/mL RNase) in PBS. The DNA content was evaluated using an EPICS XL flow cytometer (Beckman Coulter, Corp., Tokyo, Japan).

Measurement of bromodeoxyuridine uptake was done as described previously (25). Briefly, after siRNA-transfected cells and mock-transfected cells (2.0×10^6/plate) were incubated for 48 hours in serum-free medium at 37°C and 18 hours after addition of 10% FBS at 37°C, bromodeoxyuridine was added to the culture medium (10 μmol/L), and the cultures were incubated for 30 minutes at 37°C. The cells were fixed in 70% ethanol at −20°C. To denature the DNA, the cells were incubated for 30 minutes at room temperature in 2 N HCl with 0.5% Triton X-100. After neutralization with 0.1 mol/L sodium tetraborate (pH 8.5), the cells were incubated with anti-bromodeoxyuridine FITC

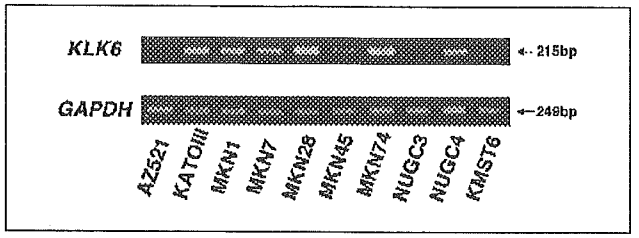

Fig. 1. Expression of *KLK6* mRNA in gastric cancer cell lines and normal fibroblast cell line. Seven of nine (78%) diverse gastric cancer cell lines are positive for *KLK6* mRNA expression, whereas nonmalignant cell lines, such as fibroblast KMST6, show a reduced expression of the gene.

(Becton Dickinson, San Jose, CA) for 30 minutes at room temperature and resuspended in 5 μg/mL propidium iodide. The cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter). These procedures were also done in triplicate.

In vitro *invasion assay.* *In vitro* invasion assays were done by using 24-well transwell units with polycarbonate filters (pore size, 8 μm) coated on the upper side with Matrigel (Becton Dickinson). Cells (5.0×10^4 cells/well) were placed in the upper chamber, and the lower chamber was filled with 750 μL of DMEM with 10% FBS as a chemoattractant. After 48 hours of incubation at 37°C, the membranes were labeled with Calcein, AM solutions. The invasive cells that had migrated through the membrane to the lower surface were read in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

Statistical analysis. The statistical analysis was done using the χ^2 method, the Mann-Whitney *U* test, Student's *t* test, and repeated measures ANOVA analysis. Survival curves were drawn according to the Kaplan-Meier method (26), and the log-rank test (27) was applied to compare the survival curves. A probability level of 0.05 was chosen for statistical significance.

Results

Clinical significance of KLK6 expression in gastric cancer: Expression of KLK6 mRNA in cell lines and clinical tissue specimens. Figure 1 shows the *KLK6* gene expression status in the human gastric cancer cell lines. Seven of the nine cell lines (78%) substantially expressed the *KLK6* gene, whereas AZ521 and NUGC3 did not. We then quantitatively determined the *KLK6* mRNA expression in clinical samples by comparison with a gastric cancer cell line, MKN28, as the quantifying standard, which expresses human *KLK6* well. Fifty-seven of the 63 patients (88%; $P < 0.0001$; Mann-Whitney *U* test) showed a higher expression level of *KLK6* mRNA in gastric cancer tissue specimens than in noncancerous tissue specimens. The mean expression value of *KLK6* mRNA in cancer tissues (0.37 ± 0.53; mean ± SD, normalized by *GAPDH* gene expression), was significantly higher than the value (0.026 ± 0.060) in the corresponding noncancerous tissues ($P < 0.000001$; Student's *t* test; Fig. 2A).

Immunohistochemistry. In 18 representative cases among the above 66 cases, we found the specific expression of hK6 protein only in cancer tissue specimens in comparison to the corresponding normal tissue specimens. In six cases with a lower expression level of *KLK6* mRNA, five cases exhibited a negative or weak expression of hK6 in cancer tissue specimens. In contrast, in 12 cases with the higher *KLK6* mRNA, 9 cases only exhibited a positive or strong hK6 protein expression in cancer tissue specimens.

The clinicopathologic significance of KLK6 mRNA expression in gastric cancer. The clinicopathologic factors analyzed are shown in Table 1 in relation with *KLK6* mRNA expression in tumor tissue. The incidence of lymphatic invasion was

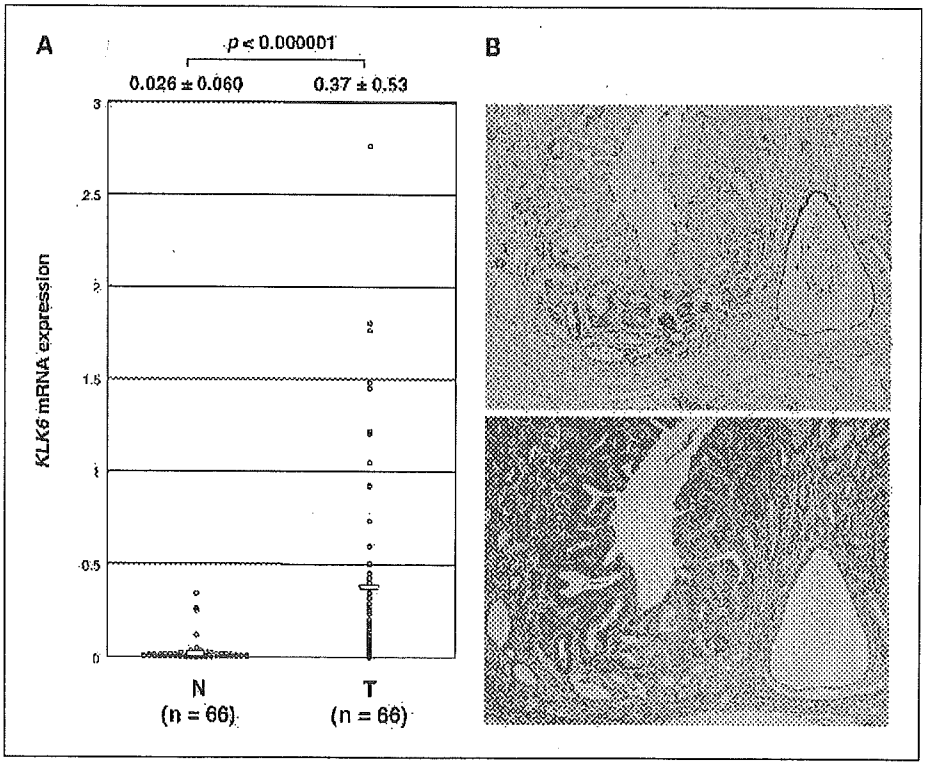

Fig. 2. Expression of *KLK6* mRNA in gastric cancer tissue and normal mucosa. *A*, the mean *KLK6* expression value in the gastric cancer tissues (0.37 ± 0.53, mean ± SD) was significantly higher than that in the corresponding normal tissues (0.026 ± 0.060). *B*, a representative gastric cancer case exhibited the KLK6 protein expression in cancer cells (top, ×40 magnification). HE staining (bottom, ×40 magnification).

Table 1. Relationships between *KLK6* status and other variables

Variables		*KLK6* Low (n = 46)	*KLK6* High (n = 20)	P*
Sex	male	29	14	n.s.
	female	17	6	
Histology	differentiated type	21	11	n.s.
	undifferentiated type	25	9	
Serosal invasion	absent	20	4	0.06
	present	26	16	
Lymph node metastasis	absent	18	5	n.s.
	present	28	15	
Lymphatic invasion	absent	19	3	0.03
	present	27	17	
Vascular invasion	absent	37	17	n.s.
	present	9	3	
Tumor-node-metastasis stage	stage I	18	4	0.03
	stage II	10	7	
	stage III	13	2	
	stage IV	5	7	

Abbreviation: n.s., not significant.
*Correlation was analyzed by the χ^2 method.

significantly higher (P = 0.03) in the high-expression group (17 of 20, 85%) than in the low-expression group (27 of 46, 59%). The incidence of serosal invasion was higher (P = 0.06) in the high-expression group (16 of 20, 80%) than in the low-expression group (26 of 46, 57%). The clinical stage also correlated with the groups (P = 0.03). On the other hand, no significant difference was observed regarding sex, histology, lymph node metastasis, and vascular invasion. The 5-year actuarial overall survival rates in patients with high *KLK6* mRNA levels and patients with low *KLK6* mRNA levels were 26% and 55%, respectively (Fig. 3). The survival difference between these two groups was statistically significant (P = 0.03; log-rank test).

Biological significance of KLK6 expression in gastric carcinoma: The KLK6 small interfering RNA–transfected gastric cancer cell lines stably suppress both KLK6 mRNA and hK6 protein. Although the highest *KLK6* expression was found in MKN74 (Fig. 1), this cell line showed an inherently low proliferation rate. Therefore, we used the MKN28 line for subsequent experiments. Among 45 stable *KLK6*-suppressed clones established using the RNA interference (RNAi) method (with *KLK6* mRNA expression <0.3 compared with MKN28 parental cells), we selected two clones (RNAi-1 and RNAi-2) and determined their expression levels of hK6 protein by Western blot analysis. These two stable *KLK6*-suppressed clones were confirmed to express markedly lower levels (about one fifth) of hK6 protein than the MKN28 parental cells (Fig. 4).

Lower proliferation activity of KLK6-suppressed cancer cells. We analyzed whether suppression of *KLK6* expression would alter the growth rate of MKN28 gastric cancer cells. As shown in Fig. 5A, there was a significant difference in growth rate

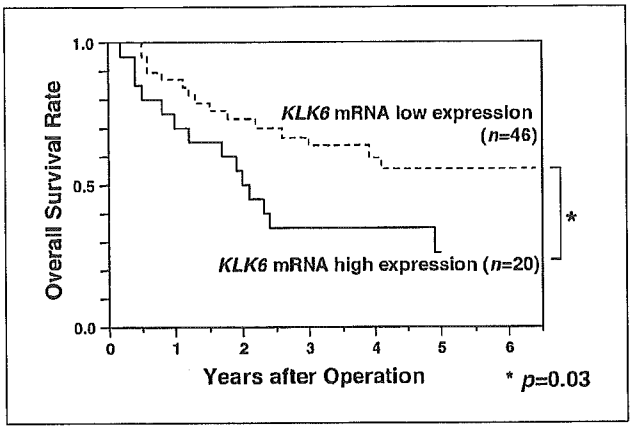

Fig. 3. Overall survival of patients with gastric cancer according to *KLK6* mRNA expression in the cancer tissue. Patients in the high *KLK6* mRNA expression group (n = 20) had a significantly poorer prognosis than those in the low *KLK6* mRNA expression group (n = 46).

between the *KLK6*-suppressed cells and the mock-transfected cells (P = 0.002). The *KLK6*-suppressed clones did not reach confluency by 10 days. To investigate whether the *KLK6*-suppressed cells showed low proliferation activity, we analyzed the cell cycle after serum starvation and after re-feeding with serum (Fig. 5B). The percentage of bromodeoxyuridine-positive cells (cells in S phase) in *KLK6*-suppressed cells was significantly lower than that in the mock-transfected cells after serum starvation for 48 hours and after addition of serum for 18 hours (P < 0.01). These results suggested that both *KLK6* mRNA and hK6 protein expression were closely associated with cell proliferation.

Low invasive potential of KLK6-suppressed cancer cells. In a clinicopathologic study, we found that the incidence of lymphatic invasion was significantly higher in the high-expression group than in the low-expression group. To verify these findings in an *in vitro* assay, we examined the invasive potential of the *KLK6*-suppressed cells using an *in vitro* Matrigel invasion assay (Fig. 6). The *KLK6*-suppressed cells exhibited significantly less invasive potential than the mock-transfected cells (P < 0.01), suggesting that high expression of *KLK6* enhanced tumor invasiveness.

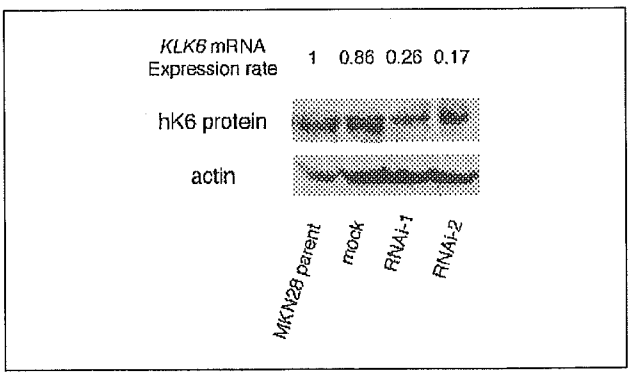

Fig. 4. Real-time reverse transcription-PCR and Western blot analysis of two *KLK6*-suppressed clones. The *KLK6* mRNA expression is about 80% decreased in both treated clones (RNAi-1 and RNAi-2). The protein levels are measured by the NIH imager with β-actin protein normalization. We also observed a reduced expression of hK6 protein in both clones.

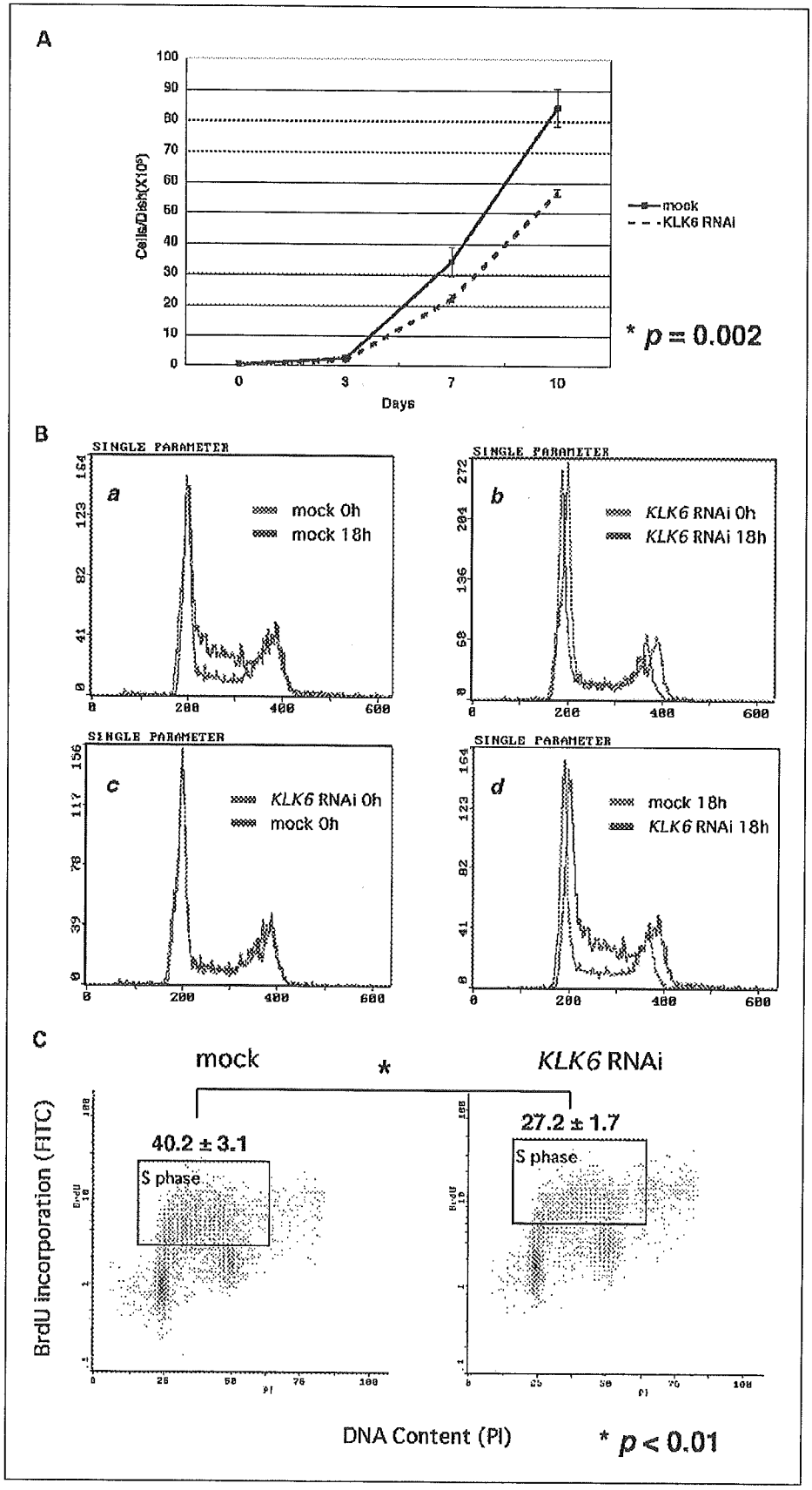

Fig. 5. Proliferation activity and cell cycling of *KLK6*-suppressed cells. The RNAi-1 clone in Fig. 4 was used for the following experiments. *A, in vitro* growth rate of *KLK6*-suppressed cells in the presence of 10% FBS. The cell number was counted on days 3, 7, and 10 in triplicate; bar, SD; $P < 0.01$; repeated measures ANOVA analysis. *B*, cell cycle of *KLK6*-suppressed cells after 48 hours of serum starvation followed by 18 hours of re-feeding with 10% FBS. (*a*) mock cells after 48 hours of serum starvation followed by 18 hours of re-feeding. (*b*) *KLK6*-suppressed cells after 48 hours of serum starvation followed by 18 hours of re-feeding. (*c*) mock and *KLK6*-suppressed cells after 48 hours of serum starvation. (*d*) mock and *KLK6*-suppressed cells 18 hours after re-feeding. *C*, the percentage of bromodeoxyuridine-positive cells in *KLK6*-suppressed cells after 48 hours of serum starvation. Each experiment was done in triplicate.

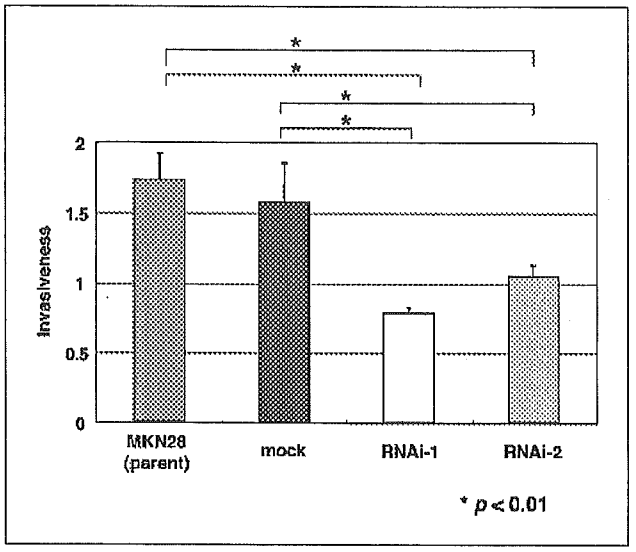

Fig. 6. Invasion potential of *KLK6*-suppressed cells. The invasive cells migrating through the membrane to the lower surface were counted by a fluorescence plate reader at excitation/emission wavelength of 485/530 nm; *bar*, SD; $P < 0.01$; Student's t test.

Discussion

In the current study, we clearly showed a sharp contrast between gastric cancer tissue and the corresponding normal counterpart with respect to *KLK6* mRNA expression, as illustrated in Fig. 2. To date, several authors have reported that *KLK6* mRNA is highly expressed in human cancer tissues (28–30). Among such gastrointestinal malignancies as colorectal cancer, Schuster et al. investigated the gene expression levels of *KLK6* by real-time reverse transcription-PCR of colorectal cancer and normal colon mucosa tissues (31). They found the mean *KLK6* expression value in normal samples to be approximately two logs lower than that of the cancer samples. Yousef et al. also reported that *KLK6* was overexpressed in colorectal cancer tissues compared with normal colon tissues (30). Moreover, our study showed a drastic change in the *KLK6* mRNA expression between the gastric cancer tissue and the normal mucosa. However, none of the previous studies investigated the clinicopathologic significance of the *KLK6* expression in cancer tissues. Thus, in this study, we examined the clinicopathologic correlation to the *KLK6* expression status in gastric cancer. The findings indicate that the overexpression of *KLK6* was significantly associated with both an increased incidence of lymphatic invasion and a poor prognosis for patients with gastric cancer. These findings suggest that an enhanced expression of *KLK6* might play an important role in various pathologic processes of gastric cancer.

To ascertain the contribution of *KLK6* to cell proliferation and invasiveness, we used MKN28 cells, which inherently expressed high levels of both *KLK6* mRNA and hK6 protein, and established the relevant suppressed clones by gene silencing using RNAi techniques. RNAi is mediated by siRNAs that are produced from the long dsRNAs of exogenous or endogenous origin by an endonuclease of the RNase-III type, called Dicer (32, 33), and has emerged as a powerful tool for understanding the gene function. With the aid of RNAi techniques, we showed that the *KLK6*-suppressed clones had markedly reduced cell growth, proliferation, and invasiveness. Regarding other *KLK*s, such as *KLK2* or *KLK3*, several reports have indicated that hK2 and hK3 might stimulate the growth and survival of tumor cells by degrading insulin-like growth factor binding proteins (IGFBP2, 3, 4, and 5), thereby liberating the mitogenic growth factor insulin-like growth factor I (IGF-I), which binds to its cell-surface receptor (IGF-IR) and induces cell proliferation and prevents apoptosis (34, 35). Furthermore, Magklara et al. reported that hK6 can degrade *in vitro* fibrinogen and collagen type I, basic constituents of the extracellular matrix, as well as collagen type IV, a major component of the basement membrane (36). Another group has also shown that hK6 can digest laminin and fibronectin in the tumor parenchyma (37). The lysis of certain components of the extracellular matrix disrupts their dynamic interactions with cells and is linked with an altered regulation of cell proliferation that can lead to tumor cell growth and malignant transformation. The results of our studies suggest that the increased hK6 expression may be associated with pericellular proteolysis and tumor invasion. Because our *in vitro* experiments showed that *KLK6* gene silencing successfully reduced tumor cell proliferation and invasiveness, siRNA-mediated gene silencing of *KLK6* might conceivably be a suitable candidate in therapy for patients with gastric cancer.

Finally, the current study indicates that *KLK6* mRNA was remarkably overexpressed in gastric cancer tissues and high *KLK6* expression levels were associated with lymphatic invasion and poor patient prognosis. hK3 has been well-documented to be an excellent tumor marker for prostate cancer. Moreover, hK6 is a promising serum biomarker for ovarian cancer. Therefore, studies are now under way to investigate whether hK6 may also be a useful biomarker for gastric cancer using serum samples from patients at our institute.

Acknowledgments

We thank Drs. Mitsuhiko Ohta, Tomoya Sudo, Kazuhiko Ogawa, Fumiaki Tanaka, and Hiroshi Inoue for important discussion. We thank M. Kasagi, M. Oda, K. Ogata, T. Shimooka, and M. Nagahara for their technical valuable assistance.

References

1. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: structure, function, and association to disease. Endocr Rev 2001;22:184–204.
2. Yousef GM, Diamandis EP. Human tissue kallikreins: a new enzymatic cascade pathway? Biol Chem 2002; 383:1045–57.
3. Diamandis EP, Yousef GM. Human tissue kallikreins: a family of new cancer biomarkers. Clin Chem 2002;48: 1198–205.
4. Borgono CA, Michael IP, Diamandis EP. Human tissue kallikreins: physiologic roles and applications in cancer. Mol Cancer Res 2004;2:257–80.
5. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 2004;4:876–90.
6. Anisowicz A, Sotiropoulou G, Stenman G, et al. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol Med 1996;2:624–36.
7. Little SP, Dixon EP, Norris F, et al. Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer's disease brain. J Biol Chem 1997;272: 25135–42.
8. Yamashiro K, Tsuruoka N, Kodama S, et al. Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim Biophys Acta 1997;1350:11–4.
9. Stamey TA, Yang N, Hay AR, et al. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 1987;317:909–16.
10. McCormack RT, Rittenhouse HG, Finlay JA, et al.

Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. Urology 1995;45:729–44.
11. Magklara A, Scorilas A, Catalona WJ, et al. The combination of human glandular kallikrein and free prostate-specific antigen (PSA) enhances discrimination between prostate cancer and benign prostatic hyperplasia in patients with moderately increased total PSA. Clin Chem 1999;45:1960–6.
12. Stenman UH. New ultrasensitive assays facilitate studies on the role of human glandular kallikrein (hK2) as a marker for prostatic disease. Clin Chem 1999;45:753–4.
13. Diamandis EP, Okui A, Mitsui S, et al. Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. Cancer Res 2002;62:295–300.
14. Luo LY, Katsaros D, Scorilas A, et al. The serum concentration of human kallikrein 10 represents a novel biomarker for ovarian cancer diagnosis and prognosis. Cancer Res 2003;63:807–11.
15. Kishi T, Grass L, Soosaipillai A, et al. Human kallikrein 8, a novel biomarker for ovarian carcinoma. Cancer Res 2003;63:2771–4.
16. Yousef GM, Polymeris ME, Grass L, et al. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res 2003;63:3958–65.
17. Borgono CA, Grass L, Soosaipillai A, et al. Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res 2003;63:9032–41.
18. Talieri M, Diamandis EP, Gourgiotis D, et al. Expression analysis of the human kallikrein 7 (KLK7) in breast tumors: a new potential biomarker for prognosis of breast carcinoma. Thromb Haemost 2004;91:180–6.
19. Diamandis EP, Yousef GM, Soosaipillai AR, et al. Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin Biochem 2000;33:579–83.
20. Diamandis EP, Scorilas A, Fracchioli S, et al. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. J Clin Oncol 2003;21:1035–43.
21. Mimori K, Mori M, Shiraishi T, et al. Clinical significance of tissue inhibitor of metalloproteinase expression in gastric carcinoma. Br J Cancer 1997;76:531–6.
22. Mori M, Staniunas RJ, Barnard GF, et al. The significance of carbonic anhydrase expression in human colorectal cancer. Gastroenterol 1993;105:820–6.
23. Ogawa K, Utsunomiya T, Mimori K, et al. Clinical significance of elongation factor-1δ mRNA expression in oesophageal carcinoma. Br J Cancer 2004;91:282–6.
24. Shibata K, Tanaka S, Shiraishi T, et al. G-protein γ7 is down-regulated in cancers and associated with p 27kip1-induced growth arrest. Cancer Res 1999;59:1096–101.
25. Mimori K, Shiraishi T, Mashino K, et al. MAL gene expression in esophageal cancer suppresses motility, invasion and tumorigenicity and enhances apoptosis through the Fas pathway. Oncogene 2003;22:3463–71.
26. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163–70.
27. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–81.
28. Tanimoto H, Underwood LJ, Shigemasa K, et al. Increased expression of protease M in ovarian tumors. Tumour Biol 2001;22:11–8.
29. Ni X, Zhang W, Huang KC, et al. Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br J Cancer 2004;91:725–31.
30. Yousef GM, Borgono CA, Popalis C, et al. In silico analysis of kallikrein gene expression in pancreatic and colon cancers. Anticancer Res 2004;24:43–51.
31. Schuster R, Max N, Mann B, et al. Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. Int J Cancer 2004;108:219–27.
32. Hammond SM, Bernstein E, Beach D, et al. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 2000;404:293–6.
33. Bernstein E, Caudy AA, Hammond SM, et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001;409:363–6.
34. Cohen P, Graves HC, Peehl DM, et al. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab 1992;75:1046–53.
35. Rehault S, Monget P, Mazerbourg S, et al. Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur J Biochem 2001;268:2960–8.
36. Magklara A, Mellati AA, Wasney GA, et al. Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors. Biochem Biophys Res Commun 2003;307:948–55.
37. Bernett MJ, Blaber SI, Scarisbrick IA, et al. Crystal structure and biochemical characterization of human kallikrein 6 reveals that a trypsin-like kallikrein is expressed in the central nervous system. J Biol Chem 2002;277:24562–70.

Clinical & Experimental Metastasis (2005) 22: 59–67
DOI 10.1007/s10585-005-4417-y

Identification of molecular markers for metastasis-related genes in primary breast cancer cells

Koshi Mimori[1,*], Akemi Kataoka[2,*], Keiji Yoshinaga[1], Mitsuhiko Ohta[1], Yasuaki Sagara[2], Yasuji Yoshikawa[3], Shinji Ohno[2], Graham F. Barnard[4] & Masaki Mori[1]

[1]*Department of Surgery Kyushu University, 874-0838 Beppu, Japan;* [2]*Department of Surgery, Kyushu National Cancer Center, Fukuoka, Japan;* [3]*Pathology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan;* [4]*University of Massachusetts Medical School, Worcester, Massachusetts, USA*

Received 26 July 2004 accepted in revised form 7 March 2005

Key words: cancer specific methylation, cDNA microarray, laser microdissection, motility related protein-1, relapse free survival

Abstract

Comparing differential gene expression profiles established by cDNA microarray between normal cells (N), primary carcinoma cells (T), and metastatic carcinoma cells (M) may determine those critical genes directly associated with progression and metastasis of breast cancer. Total RNA was extracted by laser microdissection (LMD) from 20 slices of T, N and M from 6 cases. After amplification by a T7-based system, differentially expressed genes between T, N and M were identified by cDNA microarray. In addition, to clarify the mechanism for altered gene expression, we determined the methylation status by sequencing after bisulfite treatment for intriguing genes. As a result, the expression of motility related protein-1 (*MRP-1/CD9*), peripheral myelin protein-22 (*PMP-22*), and caspase 3 (*CASP-3*) were down-regulated in M compared to T. We focused especially on *MRP-1* and found that the expression status of *MRP-1* was significantly inversely associated with stage of disease in 56 cases of breast cancer ($P < 0.05$), and the relapse free survival in 5 years was significantly higher in *MRP-1* positive cases than those negative cases ($P < 0.05$). Conversely, overexpression, by 11-fold, of signal transduction and translation factors were observed in T compared to N. The cancer specific methylation was observed only in *CASP-3* in a case. In conclusion, the establishment of the present assay allows us to detect genes directly associated with each cell population within tumor tissue and gives us clues to identify metastasis-related genes comprehensively in clinical breast cancer cases.

Abbreviations: CASP-3 – caspase 3; LMD – laser microdissection; MRP-1 – motility related protein-1; PMP-22 – peripheral myelin protein-22

Introduction

To improve the clinical outcome of breast cancer patients, it is important to prevent recurrence with distant or local metastases. However, the incidence of recurrence has not decreased despite considerable efforts. There may be unique characteristics in breast cancer cells themselves that permit a clinical recurrence more than 10 years after a curative resection. Therefore, there is a great need to further clarify the nature of breast cancer cells by identifying those important genes associated with metastasis. There have been two difficulties in determining the responsible genes, one is distinguishing cancer cells themselves from contaminating non-malignant cells in breast tissue specimens, and the second is a limitation on the number of genes that can be examined in one assay. Two powerful new tools, laser microdissection (LMD, Leica ltd. Tokyo, Japan) and cDNA microarray, may conquer these problems. For instance, our previous study using LMD demonstrated that thymidine phosphorylase (TP), previously believed to originate from colon cancer cells, actually originated from stromal, not cancer cells [1]. Furthermore, cDNA microarray enabled us to analyze the comprehensive expression profile of genes in each sample [2, 3]. The amount of extracted RNA from LMD samples is usually too small to analyze by cDNA microarray. However, another new technique, T7-based RNA amplification [4], enables us to amplify these small amounts of RNA to give sufficient cDNA for microarray analysis.

*Both authors contributed equally to this work.

Correspondence to: Masaki Mori, MD, PhD, FACS, Department of Surgery Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838, Japan. Tel: +81-977-27-1650; Fax: +81-977-27-1651; E-mail: mmori@beppu.kyushu-u.ac.jp

With this background, we first studied cases of gastric cancer by LMD and cDNA microarray [5]. This revealed that Caspase-8 and Cadherin were suppressed in primary cancer cells compared to normal cells, and conversely that matrix metalloproteinase 7 *(MMP-7)* [6–8] was overexpressed in both primary and metastatic cells in comparison with normal cells [5]. In breast cancer, a similar study, but only of a single case, was performed by previous studies [9] while Adeyinka et al. reported the gene expression profile specified by ductal carcinoma *in situ* (DCIS) [3]. Moreover, Wulfkuhle et al. reported that the proteomic analysis of matched normal ductal/lobular units and ductal carcinoma *in situ* (DCIS) of the human breast with laser capture microdissection [10].

In the present study, we identified the gene expression profile of samples obtained from primary or metastatic cancer cells and the corresponding normal cells in 6 cases of sporadic breast cancer. Moreover, another purpose of the study is to clarify a mechanism of the diminished expression of identified genes, therefore, we focused on an epigenetic alteration in identified genes.

Materials and methods

Surgical specimens from patients with breast cancer

Informed consent was obtained from all patients in our department. Six cases of sporadic breast cancer with lymph node metastasis were studied. The average age was 55.5 ranging from 37 to 69. Two cases were pre-menopausal and four post-menopausal. On histologic examination five were invasive ductal carcinoma, one was medullary carcinoma. Size ranged from 2.0 to 4.3 cm. One case was estrogen receptor negative by immunohistochemistry. Three underwent breast conserving surgery and three a modified radical mastectomy, axillary lymph node dissection was performed in all cases.

The tissue blocks from tumors, normal mammary glands, and metastatic lymph nodes were obtained at surgery from all cases. Each block was cut into two, one half was used for routine pathological diagnosis. The other half was used for molecular analysis and immediately embedded in Tissue Tek OCT compound medium (VWR Scientific Products Corporation, San Diego, CA), and frozen in liquid nitrogen. Twenty serial sections, 8 micron thick, were prepared with a cryostat, each mounted on an uncoated glass slide, and stored at −80 °C until use.

Collection of target cells by LMD from frozen sections

For the LMD, frozen section slides were fixed in 70% ethanol for 30 s, stained with H&E, and then the following dehydration steps performed, 5 s each in 70%, 95% and 100% ethanol, and a final 5 min in xylene. Once air-dried, the sections were laser microdissected with the LMD system. Target cells were cut out, at least 100 cells per section, and bound to the transfer film. We treated 20 sections of every sample, thus about 10,000–20,000 cells were collected for the total RNA extraction. Total RNA was extracted using ISOGENE (Nippon Gene, Japan) according to the manufacturer's instructions.

Amplification of mRNA from each target

First strand synthesis: mix mRNA, 9 μl and oligo-dT T7 primer, 5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T15–3′, in a 0.2 ml RNase-free tube, heat at 65 °C for 10 min. then put on ice. Add 4 μl 5× first strand buffer, 2 μl 0.1 M DTT (Gibco BRL, Japan), 1.3 μl 10 mM dNTP mix (Pharmacia (27-2035-01) and 1 μl Superscript II (Gibco BRL (18064-014), incubate at 42 °C for 60 min then place on ice. Second strand synthesis: add the following solutions on ice to the first strand reaction: 106 μl RNase free water, 15 μl 10× second strand buffer, 3 μl 10 mM dNTP mixture (dilute in DEPC water, Pharmacia), 1 μl *Escherichia coli* DNA ligase, 4 μl *E. coli* DNA polymerase I-holoenzyme (19 U/μl, NEB (209L) and 1 μl RNase H (2 U/ml, GibcoBRL (18021-071). Incubate at 16 °C for 2 h. Then perform an additional second strand synthesis by adding repairing enzyme mixture: 1.68 μl Ex Taq (5 U/μl, Takara), 0.25 μl Ampligase (Epicenter), 0.12 μl Hybridase (Epicentre); incubate the solution at 65 °C for 30 min. Stop the reaction with 10 μl 0.5 M EDTA and incubate at 65 °C for 10 min. Sample extraction and precipitation: Carry out phenol chloroform (1:1) extraction twice. Add organics directly to PCR tubes, mix by pipet, and spin 5 min at maximum speed at room temperature. Transfer aqueous phase to RNase free 1.5 ml Eppendorf tubes. Repeat chloroform extraction twice. Add 70 μl 7.5 M ammonium acetate to the aqueous phase, and add 1 ml pure ethanol, store at −20 °C for ethanol precipitation. This is the end of the first step, we repeated these amplification procedures two more times. We amplified RNA successfully with an estimated 10^4-fold amplification using this T7 RNA polymerase method.

Expression profiling of cancer-related genes by cDNA microarray

Three μg aliquots of mRNA from cancer tissue and the corresponding normal tissue were labeled with Cy5-dCTP and Cy3-dCTP (Amersham, Biotech), respectively. In addition, cluster of metastatic cancer cells and that of the corresponding primary cancer cells were also labeled, respectively with Cy3-dCTP and Cy5-dCTP mixed with the RNA Fluorescence Labeling Core Kit (Takara, Japan). Labeled probes were hybridized with 624 genes on the Takara human Cancer Chip version 2.0 (Takara, Japan) in hybridization buffer (Takara, Japan) for 14–16 h at 65 °C. After hybridization, the slides were washed twice in 2× SSC/0.2% SDS for 30 min at 55 °C and 2× SSC/0.2% SDS for 30 min at 65 °C, then in

0.05× SSC for 5 min at room temperature. Immediately, scanning of slides was performed by the GMS418 Array Scanner (Genetic Microsystems, Japan). The intensity of each hybridization signal was evaluated by the computer program, ImaGene version 3.0 and normalization of expression was performed with housekeeping genes on the Chip. In each case we evaluated the expression profiles between primary cancer cells and the corresponding normal cells, and between primary cancer cells and the metastatic cancer cells in the lymph nodes.

Confirmation of the relationship between gene expression and clinicopathologic variables by RT-PCR in 56 breast carcinoma cases

Two μg of total RNA was reverse-transcribed using the same gene-specific primers as chosen for constructing the microarray. Information on the primer sequences is available commercially from TaKaRa Shuzo Co. (Shiga, Japan). RT-PCR reactions were performed for each case and the products electrophoresed in 2.0% ethidium bromide agarose gels. The expression ratio was calculated using NIH image (Macintosh software, version 1.62).

In order to confirm the concordant association between data from microarray analysis and RT-PCR, we performed RT-PCR for representative genes. Those representative genes were apoptosis inhibitor 4 (survivin), cadherin-3 (*CDH-3*), a disintegrin and metalloproteinase domain 9 (*ADAM-9*), peripheral myelin protein-22 (*PMP-22*), and Caspase-3 (*CASP-3*) and the amplified primers were as follows; 5′-CCACCG CATCTCTACATTCA-3′ (forward) and 5′-TAT GTTCCTCTATGGGGTCG-3′ (reverse) for survivin; 5′-ACGGCAGAGGTCAACGAGGAAG-3′ (forward) and 5′-GGCGGGGCTGTGGGGTCTGT-3′ (reverse) for *CDH-3*; 5′-TGGAACTGCAGGAATGGCA-3′ (forward) and 5′- CCAAACACATTAATCCCGCC-3′ (reverse) for *ADAM-9*; 5′-GTCTCCACCATCGTCA GCCAATG-3′ (forward) and 5′-CTCATCACGCA CAGACCAGCAAG-3′ (reverse) for *PMP-22*; and 5′-GGAAGCGAATCAATGGACTCT-3′ (forward) and 5′-ACGGCAGGCCTGAATAATG-3′ (reverse) for *CASP-3*.

Among representative those genes, we focused on motility related protein-1 (*MRP-1/CD9*), since this gene showed the greatest diminution in expression in metastatic cancer cells compared to the corresponding primary cancer cells. We examined the expression of *MRP-1/CD9* in 56 cases of breast cancer surgically removed from 1998 to 2002 in our department with adequate informed consent. We compared it with the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by RT-PCR in primary cancer tissues of each case. The oligonucleotide primer pairs for *MRP-1* were as follows; 5′-TGCATCTGTATCCAGCGCCA-3′ (forward) and 5′-CTCAGGGATGTAAGCTGACT-3′ (reverse). The amplification was performed for 30 cycles of 40 s at 94 °C, 40 s at 60 °C, and 90 s at 72 °C. The cases with values equal or more than 0.1 were considered positive, and those with values of less than 0.1 as negative [11]. The centers of the carcinoma and normal tissues were extracted from the surgical resected specimens and diagnosed pathologically. All the samples were stored immediately at −80 °C until used and were handled carefully so as not to contaminate with sources of RNAase. The average patient age was 56 years and ranged from 27 to 77 years. The data of clinicopathological variables such as lymph vessel invasion, vascular vessel invasion, lymph node metastasis, estrogen or progesterone receptor status and TNM classification and 5 year relapse free survival rate were reviewed for all patients. Among 59 cases of breast cancer, the adjuvant chemotherapies, such as CE (Cyclophosphamide + Epirubicin hydrochloride) or CMF (Cyclophosphamide + Methotrexate) were performed for 22 cases of lymph node metastasis positive, while adjuvant hormone therapies including Tamoxifen citrate or Anastrozole were applied for 35 cases of either estrogen receptor positive or progesterone receptor positive.

Bisulfite treatment and sequencing

Genomic DNA was extracted using the QIAGEN DNA extraction Midi Kit (QIAGEN, Tokyo Japan) from tumor and corresponding normal tissues in the 6 cases, 1 μg of the DNA was subjected to bisulfite treatment. These tissues were adjacent to the slices taken for the LMD study. For DNA denaturation, 2 μg of the DNA were used and water added to 20 μl. Salmon sperm DNA (5 μg) and 2 μl of 3 M NaOH were added to the mixture, that was then heated for 30 min at 50 °C. For preparation of the bisulfite solution, we put water for hydroquinone and water for bisulfite in a 50 °C water bath. NaOH (2M) and bisulfite were added to the water that was left to mix at 50 °C. Hydroquinone was added to the second warm water to make a 1 M solution, and the mixture was well dissolved. The dissolved hydroquinone was added to the bisulfite mixture.

For the bisulfite reaction, 500 μl of the Bisulfite/Hydroquinone solution were added to the denatured DNA, and the mixture was incubated for 1 h at 70 °C At the end of the reaction we used a Wizard DNA Clean-Up KIT. First we prepared 80%- isopropanol and warm H_2O (80 °C) in a water bath, then we added 1 ml of Resin from the KIT to the column. After mixing the sample well it was put onto the column, a vacuum applied, 2 ml isopropanol were added in a syringe, and the vacuum re-applied (2 times). Then, the filter was placed into a clean 1.5 ml tube and centrifuged at maximum speed for 2 min to remove all isopropanol. We added 45 μl H_2O (80 °C), waited for 1 min, then spun down to maximize yield. After a 1 min spin at full speed, 5 μl of 3 M NaOH were added, and the tube incubated for 10 min at room temperature for deamination. Ethanol precipitation was performed with 75 μl of 5 M ammonium acetate as usual.

Direct sequencing of bisulfite-treated genomic DNA for genes of interest. According to the listed genes, we

identified and focused on motility related protein-1 (*MRP-1*), peripheral myelin protein-22 (*PMP-22*), and Caspase-3 (*CASP-3*) as potential suppressor genes in lymph node metastasis of breast cancer. We determined the sequence of the potential promotor region located upstream from the 1st nucleotide of exon 1, after bisulfite treatment of the genomic DNA from primary cancer tissues and corresponding normal tissues.

Results

We made 20 frozen sections from each primary tumor, normal mammary gland or metastatic lymph node for all 6 cases. As there are typically more than 100 cells in a cancer nest, we extracted 300–800 cells from each slide section as shown in Figure 1. After 3 rounds of T7 amplification, an approximately 10^4 fold mRNA amplification from each sample is achieved.

Genes identified with reduced expression in metastatic cells compared to corresponding primary carcinoma cells

The average expression ratio of genes in metastatic cancer cells relative to primary cancer cells (M/T) was calculated as 0.96 (range 1.87–0.12). We identified *MRP-1*, peripheral myelin protein-22 (*PMP-22*) and caspase-3 (*CASP-3*) as genes with repressed expression in metastatic breast cancer cells compared to the corresponding primary tumor cells (Figure 2A).

Genes overexpressed in metastatic cancer cells compared to corresponding primary carcinoma cells

Conversely, the expression of 3 genes, a disintegrin-metalloproteinase domain 9 (*ADAM-9*) [12], *BCL-2* [13–15] and early growth response-1 [16] was notably higher in metastatic cancer cells than in the corresponding primary cancer cells (Figure 2B).

Genes identified by comparing primary cancer cells with the corresponding normal cells

Expression profiles of genes from primary cancer cells were also compared with the normal adjacent mammary gland tissue cells. The expression levels of *PMP-22* and *CASP-3* in the primary cancer cells was less than half of that in the normal cells (Figure 2c). While the expression ratio of *MRP-1* between primary cancer cells and the corresponding normal cells was less than 1 (data not shown).

The average expression ratio of genes in primary cancer cells relative to normal cells (T/N) was calculated as 1.1 (range from 11.7 to 0.23, Figure 2D). We observed 8 genes, such as signal transducer and activator of transcription 1 (*STAT-1*), eukaryotic translation initiation factor 2 (*eIF-2*), and cell division cycle 2 (*cdc-2*) that were overexpressed more than 2 fold in the cancer cells compared to the normal cells. The putative functions of these genes are critical for progression of cancer cells, such as those relating to cell cycle regulation, growth factors, proteinases, or signal transduction systems.

In order to confirm the general reproducibility of the intensities of representative genes determined by microarray, we performed conventional assays by RT-PCR to compare M/T and T/N samples and we found a relative concordant relationship between microarray and RT-PCR (Figure 3).

Clinical significance of the altered expression of MRP-1 in breast cancer cases

As we previously identified *MRP-1* as a potential key molecule for cancer metastasis in colorectal cancer [11], we focused on the gene and examined its clinical significance in primary breast cancer cases [11]. In 56 breast cancer cases, *MRP-1* expression could be clearly classified into two groups, 15 cases with *MRP-1* positive expression (+) and 41 cases with negative expression

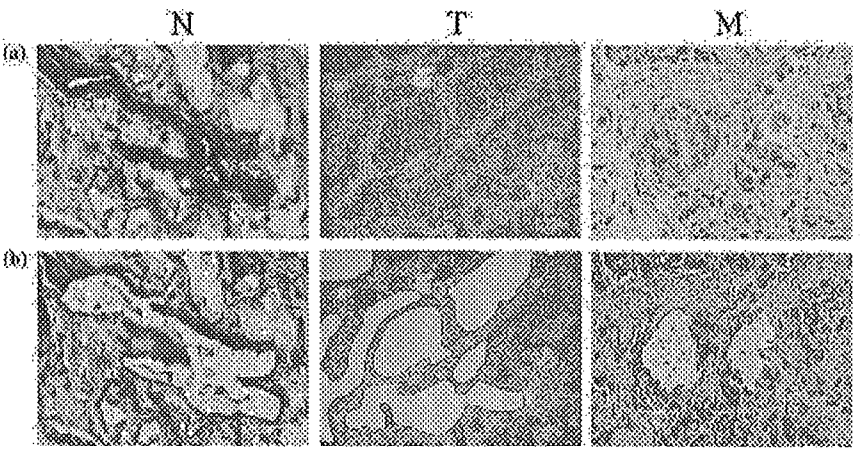

Figure 1. A representative case of laser microdissection (LMD). Sections before (a) and after (b) microdissection of normal (N), primary tumor cancer (T), and lymph node metastatic (M) cells.

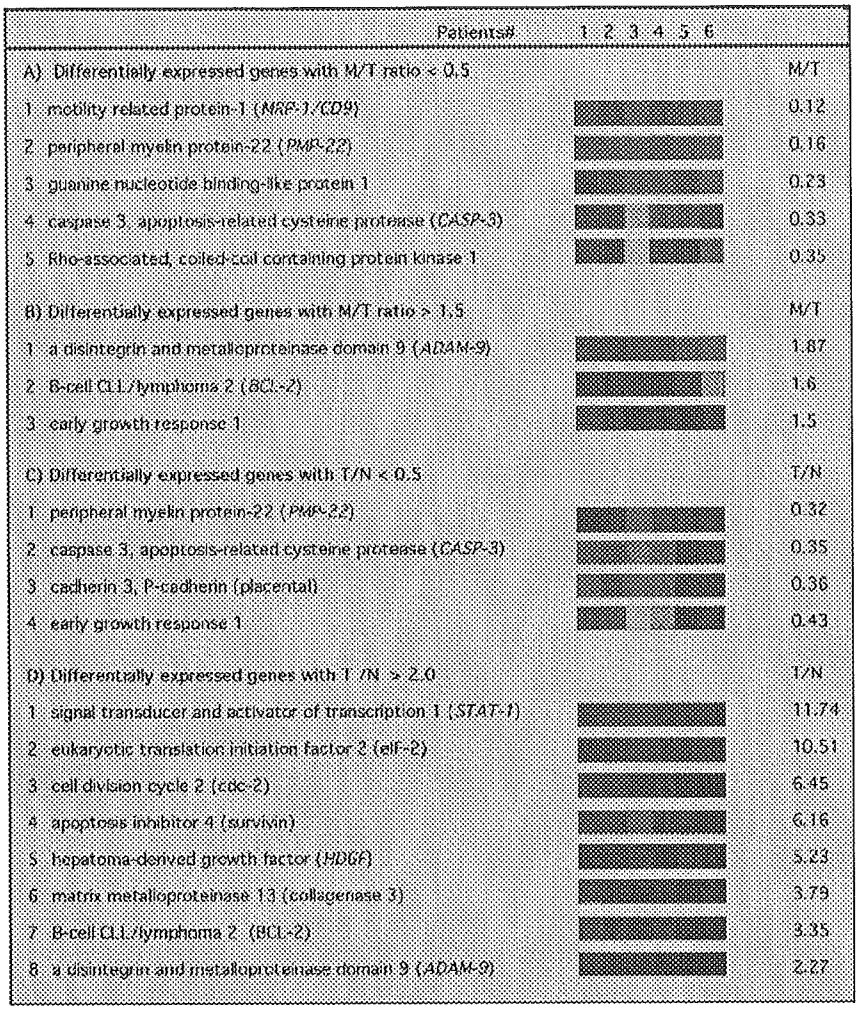

Figure 2. List of differentially expressed genes among metastatic cancer cells (M), primary cancer cell (T) and normal cells (N). The higher expressions of the reference is red, while reduced expression is green. Cancer cells from the metastastic tissue and that from primary tumor tissue are compared in (A) and (B), on the other hand, cancer cells from primary cancer and the corresponding normal cells are compared in (C) and (D).

(−). Among clinicopathologic variables, 19 of 21 (90%) of *MRP-1* (+) cases were grouped as low stage (stage I or II) while 22 of 35 (63%) *MRP-1* (−) cases belonged to a higher stage group (stage III) (Table 1). There was a statistically significant difference between stage of disease and the status of *MRP-1* expression ($P < 0.05$). In addition, after 5 years follow up, relapse free survival rate was higher in 15 cases of *MRP-1* (+) than 41 cases of *MRP-1* (−) (Figure 4). However, there was no significant difference in overall survival rate because of the small number of cases (data not shown). Furthermore, the influence of adjuvant for the expression of *MRP-1* was unknown.

One mechanism of the diminished gene expression

We examined the methylation status of *MRP-1*, *PMP-22* and *CASP-3* to elucidate possible mechanisms for the diminished expression of those representative genes in breast cancer tissue. Among 6 examined cases, cancer specific methylation of *CASP-3* was observed in a case (17%). Figure 5 shows a representative case exhibiting cancer specific methylation compared to the corresponding normal tissue in a primary breast cancer. On the other hand, there is no methylation in cancer cells nor normal cells in *PMP-22* and *MRP-1* genes (data not shown).

Discussion

Up-regulated or down-regulated genes described here in comparisons of normal, primary and metastatic cancer cells may reflect progression to the final phenotype in the evolution of the metastatic process. The application of new technologies in the present study provides clues to help dissect the complexities in the mechanism of metastasis in breast cancer. From the listed genes in Figure 2, we discuss several representative ones.

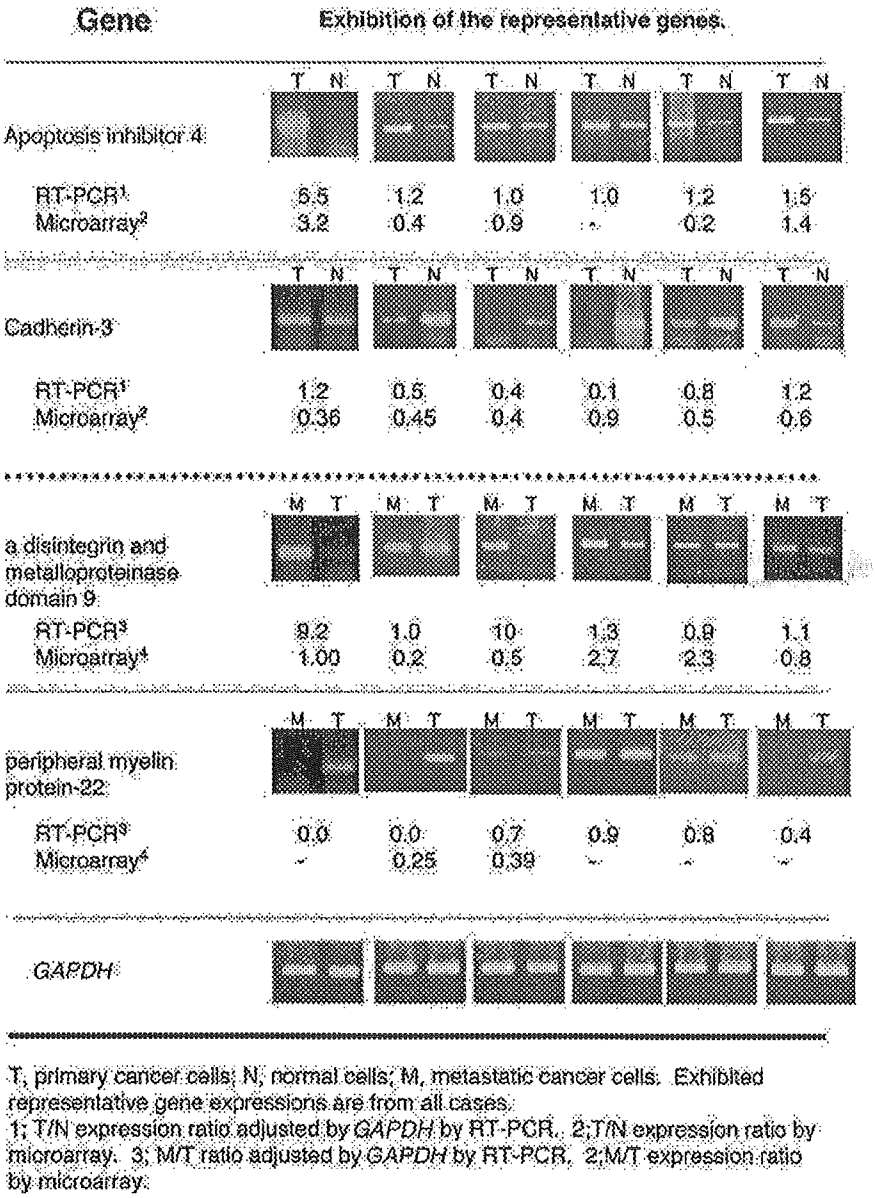

Figure 3. Comparison data of M/T and T/N ratios obtained by microarray analysis and semi-quantitative RT-PCR assays in 6 breast cancer cases. Two representative genes on the upper rows illustrate differences between primary cancer cells (T) and normal cells (N); Two representative genes on the lower rows illustrate differences between metastatic cancer cells (M) and primary cancer cells (T). Equal expression of a control gene (glyceraldehydes-3-phosphate dehydrogenase) was confirmed for the T, N, and M samples. The ratios calculated by the microarrays were presumed to be generally reliable given the demonstration of fairly consistent results based on the RT-PCR assays.

Firstly, among the genes diminished in metastatic cancer cells compared to primary cancer cells, the expression of *MRP-1* was distinctive. Thus, we consider that *MRP-1* may be a key molecule whose diminished expression contributes to the lymph node metastasis of breast cancer cells. *MRP-1* is known as a membrane glycoprotein identical to the CD9 antigen (24 kD) and it belongs to a structurally distinct family of cell membrane glycoproteins. As for relationship of *MRP-1* with malignancies, the reduced *MRP-1* expression was significantly associated with high grade and lower disease-free survival in 153 cases of head and neck cancer and 111 cases of urothelial bladder carcinoma [17, 18]. In addition, Hashida et al. described that the overall survival rate of patients with *MRP-1*-positive tumors was much higher than that of patients with *MRP-1*-negative tumors in 146 cases of colorectal cancer. Alterations of cell membrane glycoproteins may be associated with tumor cell progression and metastasis [19]. Several possible functions have been reported for reduced *MRP-1* expression may evade control by the immune system because its expression closely correlated with that of MHC class I antigen [20]. As we expected, we found a significant association between lack of *MRP-1*

Table 1. Clinicopathologic features of the expression of *MRP-1/CD9* mRNA in 56 cases of breast carcinoma.

Variables	*n*	*MRP-1* expression*		*P*-value
		Negative (41 cases)	Positive (15 cases)	
Lymph vessel invasion				ns
Absent	36	26	10	
Present	20	15	5	
Vascular vessel invasion				ns
Absent	42	31	11	
Present	14	10	4	
Lymph node metastasis				ns
Absent	34	24	10	
Present	22	17	5	
Estrogen receptor				ns
Present	22	18	4	
Absent	34	23	11	
Progesteron receptor				ns
Present	20	12	8	
Absent	36	29	7	
TNM stage				$P < 0.05$
0, I and II	35	22	13	
III	21	19	2	

*The expression of *MRP-1* was corrected by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in each cases. Values of equal or more than 0.1 were considered positive and those with values of less than 0.1 as negative.

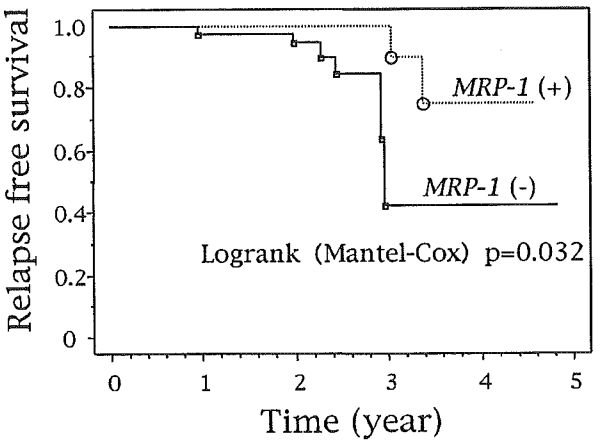

Figure 4. Relapse free survival in *MRP-1* positive and negative cases. *MRP-1* positive cases showed significantly higher relapse free survival rate than *MRP-1* negative cases by Logrank (Mantel–Cox) test ($P < 0.05$).

expression and the stage of disease, however in our present group of 56 cases there was no statistically significant difference between diminished *MRP-1* expression and the incidence of lymph node metastasis specifically. Nonetheless, others have reported a reduced *MRP-1* expression in breast cancer that was significantly associated with lymph node metastasis and a worse prognosis [21, 22]. We cannot fully explain this discrepancy, however, one most important finding from

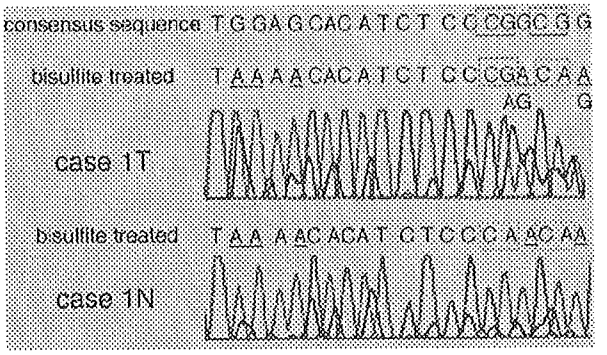

Figure 5. Promotor methylation of the CpG island of Caspase 3 (*CASP-3*) after bisulfite treatment of cancer and corresponding normal tissues. In one out of 6 cases, the CpG island located upstream from the 1st nucleotide of exon 1 is methylated in the cancer tissue, however, the identical position is not methylated after the bisulfite treatment in the corresponding normal tissue.

our study is that we could confirm the correlation between *MRP-1* and the relapse free survival using the comprehensive analysis of many genes on microdissected cells without contamination of interstitial cells.

In addition, we identified *PMP-22* and *CASP-3* as typically having diminished expression in comparisons of both primary tumor cells with normal cells and of metastatic cells with primary tumor cells. It is worth pointing out that this is the first report to describe the diminished expression of *PMP-22* in breast cancer cells. With respect to the mechanism of repression of this gene, Hildebrandt et al. reported that melanomas showed LOH for the *PMP-22* gene in 10–20% of primary tumors, and 50% of melanoma metastases [23]. However, we could not indicate any clinical significance of *PMP-22* and *CASP-3* in breast cancer cases by real time RT-PCR assay. This is because we have used mRNA not from the laser microdissected tissues, but from bulk tissues of 56 cases.

Secondly, comparison data for gene expression profiles of primary cancer cells and the corresponding normal cells is listed in Figures 2c and d. We anticipated that these genes would be involved in carcinogenesis or tumorigenesis rather than tumor spread. In fact, they can be classified mainly into the following functional groups: signal transduction or translation related genes (*STAT-1* [24], *eIF-2* [25]), cell cycle related genes (*cdc-2* [26]), apoptosis related genes (*surviving* [27], *Bcl-2* [28]), growth factors (*HDGF* [29]) and protease related genes (*MMP-13* [30], *ADAM-9* [12]). In particular, with reference to *STAT-1*, cells are programmed to self-destruct when they become abnormal such as in a cancer, thus *STAT-1* interferes with the process that immortalizes cells and initiates their out-of-control proliferation [31]. Widschwendter et al. have taken a position against a powerful role of *STAT-1* in malignancy [24]. They reported that *STAT* was activated in mammary carcinoma, and that patients with high *STAT-1* activation had a substantially longer overall and relapse-free survival.