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Advances in Brief

Differential Gene Expression Profiles of Scirrhous Gastric Cancer Cells with High Metastatic Potential to Peritoneum or Lymph Nodes[1]

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Abstract

Scirrhous gastric cancer is often accompanied by metastasis to the peritoneum and/or lymph nodes, resulting in the highest mortality rate among gastric cancers. Mechanisms involved in gastric cancer metastasis are not fully clarified because metastasis involves multiple steps and requires the accumulation of altered expression of many different genes. Thus, independent analysis of any single gene would be insufficient to understand all of the aspects of gastric cancer metastasis. In this study, we performed global analysis on differential gene expression of a scirrhous gastric cancer cell line (OCUM-2M) and its derivative sublines with high potential for metastasis to the peritoneal cavity (OCUM-2MD3) and lymph nodes (OCUM-2MLN) in a nude mice model. By applying a high-density oligonucleotide array method, expression of approximately 6800 genes was analyzed, and selected genes were confirmed by the Northern blot method. In our observations in OCUM-2MD3 cells, 12 genes were up-regulated, and 20 genes were down-regulated. In OCUM-2MLN cells, five genes were up-regulated, and five genes were down-regulated. The analysis revealed two functional gene clusters with altered expression: (*a*) down-regulation of a cluster of squamous cell differentiation marker genes such as small proline-rich proteins [SPRRs (SPRR1A, SPRR1B, and SPRR2A], annexin A1, epithelial membrane protein 1, cellular retinoic acid-binding protein 2, and mesothelin in OCUM-2MD3 cells; and (*b*) up-regulation of a cluster of antigen-presenting genes such as MHC class II (DP, DR, and DM) and invariant chain (Ii) in OCUM-2MLN cells through up-regulation of CIITA (MHC class II transactivator). We then analyzed six gastric cancer cell lines by Northern blot and observed preferential up-regulation of trefoil factor 1, α-1-antitrypsin, and galectin 4 and down-regulation of cytidine deaminase in cells prone to peritoneal dissemination. Genes highly correlated with invasion or peritoneal dissemination of gastric cancer, such as E-cadherin or integrin β_4, were down-regulated in both of the derivative cell lines analyzed in this study. This is the first demonstration of global gene expression analysis of gastric cancer cells with different metastatic potentials, and these results provide a new insight in the study of human gastric cancer metastasis.

Introduction

Gastric cancer has been a major cause of cancer death (1). Scirrhous gastric cancer, a diffusely infiltrating type of poorly differentiated gastric cancer, affects younger patients and has the highest mortality of all gastric cancers. Common features of scirrhous gastric cancer include invasive progression, remarkable fibrosis, and a high frequency of metastasis to peritoneum or lymph nodes (2). Among these malignant characteristics of scirrhous gastric cancer cells, metastasis to the peritoneum or lymph nodes is an especially complex phenomenon that requires the involvement of many different genes in multiple steps for both tumor cells and the surrounding stromal cells.

Although aspects of gastric cancer metastasis remain to be elucidated, adhesion molecules have been reported to play a pivotal role. For example, decreased expression of E-cadherin, which mediates cell to cell adhesion, is correlated with the metastatic or invasive phenotype of gastric cancer cells (3). Another adhesion molecule, integrin, which mediates cell to cell and cell to matrix adhesion, has also been known for its involvement in the peritoneal dissemination of gastric cancer cells; the β_1 subunit of integrin was reported to be a promoter (4), and the β_4 subunit was reported to be a suppressor (5). Apoptosis-related genes such as BAG-1, the Bcl-2 family gene, were reported to promote peritoneal dissemination of gastric cancer cells through an antiapoptotic effect (6). α-1,3-Fucosyltransferase modifies cell surface glycan and facilitates cancer cell adhesion to the peritoneum via the E-selectin system (7).

Previously, Yashiro *et al.* (8) and Fujihara *et al.* (9) have established OCUM-2MD3 cells (8), which have a high potential for peritoneal dissemination when injected i.p. in a nude mice model, and OCUM-2MLN cells (9), which have a high potential for metastasis to lymph nodes when implanted orthotopically in a nude mice model, from scirrhous gastric cancer cell line OCUM-2M to investigate the mechanism of gastric cancer metastasis. OCUM-2MD3 and OCUM-2MLN cells are the established models to study peritoneal dissemination and lymph node metastasis, respectively, and have been studied extensively both *in vitro* and *in vivo*. This model has been applied to describe the promotive effects of CD44H (10), integrin β_1 (4, 11), and factors produced by cocultured fibroblasts (12) in peritoneal dissemination.

Multiple genes are involved coordinately in metastasis, whereas only one gene or a few genes have been considered in most previous reports on metastasis. Moreover, differences in metastatic potential are expected to be due to a combination of differently expressed genes. In this context, gene expression analysis of scirrhous gastric cancer cells with different metastatic potentials in terms of grade and target is extremely relevant to clarify the mechanism of gastric cancer metastasis. In this study, we globally analyzed expression profiles of approximately 6800 genes in OCUM-2M, OCUM-2MD3, and OCUM-2MLN cells by using a high-density oligonucleotide array. By combining this analysis with subsequent confirmation of altered expression in selected genes by Northern blot analysis, we identified differently expressed genes among these cells. Although further functional analysis is necessary, these results will give new insight on the elucidation of the mechanism of gastric cancer metastasis.

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Table 1 *Primer pairs used in RT-PCR*

Gene	Sense (5′→3′)	Antisense (5′→3′)
37-kDa LIM domain protein	ATGACACACCTGGAGGCACAGAAC	AAAGAAGTAACCACGCTGCTTGAG
α (1,3)-fucosyltransferase	TCAAGACGATCCCACTGGTACC	GGAACCTCTCGTAGTTGCTTCTG
α-1-antitrypsin	GCCCAGAAGACAGATACATCCCAC	CATGCCTAAACGCTTCATCATAGG
Annexin A1	CCTTGAGGAGGTTGTTTTAGCTCTG	GCGAGTTCCAACACCTTTCATG
Bik	TGGGTCTGGCTTTCATCTACGACC	CCGAGGGATCAACATATCACACAG
CD81	AAGACCTTCCACGAGACGCTTG	TGTGACATCAGCCCTGAAAAGG
CIITA	CTCCCGAGCAAACATGACAGAG	CAAGATGTGGCTGAAAACCTCATC
CRABP2	CATCAAAACCTCCACCACCGTG	TTTGGGGCTAGGACTGCTGACTTG
Cytidine deaminase	CAACATAGAAAATGCCTGCTACCC	GAAAAGTTGCTCGGAACAGGATAG
Defensin β2	AGCTCCCAGCCATCAGCCATGAG	CTCTGATGAGGGAGCCCTTTCTG
Dodesenoyl-CoA isomerase	ATTCAAGAACCCCCCAGTGAAC	TGGAGATGAAGCTGACGAAGTTC
E-cadherin	ACAGGATGGCTGAAGGTGACAGAG	CTTCATTCACATCCAGCACATCCAC
E48	GACATCAGAGATGAGGACAGCATTG	CGGCAACAAAACAGAAGGAGTG
EphA1	GATGGGTGGAGTGAACAGCAAC	GACAATGGGGTGTGTCCATGTC
EMP1	GAGAGTTTGTAAGGTCATGCTGGTG	ATTTTCCTGGGAATGCAGGCAC
G3PDH	AATGCCTCCTGCACCACCAACTGC	CATGTGGGCCATGAGGTCCACCAC
GA733-1	CACCCACCACATCCTCATTGAC	TCTCAACTCCCCCAGTTCCTTG
Galectin 4	GCCTTTGAGCTGGTCTTCATAGTC	CCAAATGGGTTGTGGGTGATC
GS1	CCCACCTCATCTTTGACATGGAC	GCTCAAGTTTCCGTCAGGAACC
Heparin cofactor 2	CAATCTAGTGGAGTCCCTGAAGTTG	CAAGAATGGTGCTAGTTGTGTACCC
IGFBP2[a]	GCACATCCCCAACTGTGACAAG	AAGAAGGAGCAGGTGTGGCATC
IGFBP6	ACAGGATGTGAACCGCAGAGAC	AAGCACAGCTTTATTGACACCCC
Integrin β_4	ATCAACTACTCGGCGATCCACC	ACTTGGTCTGCTGGAGCTTGTG
Invariant chain	GCAAAAGGCAGTTCCACCAAAG	TACACCTTCAGGGGGTCAGCATTC
IQGAP1	CTAACAGAGCTTGGAACCGTGG	TGAGAAGGAAGATCAGGAGGTTGAC
Laminin γ1	GACCCTTAGCTCCAGTTTTCTTCTG	GCTGCAAACACAAGGCACAATTC
Mesothelin	TGGGCTACCTCTTCCTCAAGATG	CCATGCTCACATTCTGCTGACTG
MHC class II DM α	TGTTACCACTTCTGTGGCTGCTAC	TTCGTGAGTCACAATGCAGGAG
MHC class II DM β	CGCAATGGGCTTCAGAATTGTG	TCTCACTTGGAGTGGAAGTTGTAGG
MHC class II DP α	AACACCCTCATCTGCCACATTG	GCAATTCAGTCAGCCACTGGAGTAG
MHC class II DR α	ATATGGCAAAGAAGGAGACGGTC	CCCTTGATGATGAAGATGGTCCC
MHC class II DR β	GCCTGATCCAGAATGGAGATTG	GTTTGTCGTACAGGGTGAGGTACAG
MHC class IA	AGATAATGTATGGCTGCGACGTG	TGCTGCACATGGCAGGTGTAC
MHC class IB	GAGGTATTTCTACACCGCCATGTC	GAAGGTTCTATCTCCTGCTGGTCTG
Na/K/Cl transporter	TCTACCCAGCTTTCCAGATGTTTG	GTGAGTTTGGAGCACCTGTCATAAC
Polymeric-immunoglobulin receptor	GCTACGTCTCCAGCAAATATGCAG	GGTCTTCATAAACCAGCTCGGG
Protein kinase C-like 1	TCATTTTGGGAAGGTGCTCCTC	TCGAAGACCTCCTCCTCATCATC
rab32	AGGAGCGATTTGGCAACATGAC	CCAGCTAACATGACATCTGTAACCC
S100P	TGAATCTAGCACCATGACGGAAC	AATCAGAGGTACATGAGCAGGCTC
SPRR1A	TCAGCAGCAGAAGCAGCCTTGC	TGCTGGAGTGACCGTTGAAGG
SPRR1B	CCACCAGTTCTAAGGGACCATACAG	GCACATTCTTTTTTGGGGGAGAC
SPRR2A	ATGTCTTATCAACAGCAGCAGTG	TTACTTGCTCTTGGGTGGATAC
Sulphotransferase monoamine preferring	GTGCCCTTCCTTGAGGTCAATG	TGCCATCTTCTCCGCATAGTCC
Trefoil factor 1	TTGGAGCAGAGAGGAGGCAATG	CAGAGCAGTCAATCTGTGTTGTGAG
Trefoil factor 3	ACGGTGGTCATGGCTGCCAGAG	TGCCTCAGAAGGTGCATTCTGC

[a] IGFBP, insulin-like growth factor binding protein.

Materials and Methods

Cell Culture and RNA Preparation. OCUM-2M, OCUM-2MD3, and OCUM-2MLN were established previously by Yashiro *et al.* (8) and Fujihara *et al.* (9). GT3TKB, MKN7, MKN45, and MKN74 were purchased from Riken Cell Bank (Tsukuba, Japan). NUGC-3 and NUGC-4 were purchased from Health Science Research Resources Bank (Osaka, Japan). OCUM-2M, OCUM-2MD3, OCUM-2MLN, and GT3TKB were maintained at 37°C in a humidified atmosphere of 5% CO_2 in high-glucose DMEM (Sigma, St. Louis, MO), whereas MKN7, MKN45, MKN74, NUGC-3, and NUGC-4 were maintained in RPMI 1640 (Sigma). Both media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin. When they reached 80–90% confluence, cells were washed with ice-cold PBS and homogenized immediately in Isogen reagent (Nippon Gene, Osaka, Japan). Total RNA was extracted according to the manufacturer's instructions. Polyadenylated RNA was purified from total RNA by Oligotex-(dT)30 mRNA purification kit (Takara, Osaka, Japan) and enriched by Microcon-100 (Millipore, Bedford, MA).

High-Density Oligonucleotide Array Analysis. One μg of polyadenylated RNA from OCUM-2M, OCUM-2MD3, and OCUM-2MLN was amplified up to approximately 100 μg of cRNA and hybridized to the high-density oligonucleotide array (GeneChip HuGeneFL array; Affymetrix, Santa Clara, CA) as described previously (13). Intensity for each feature of the array was calculated by using Affymetrix GeneChip version 3.3 software with class ABC mask file. This so-called mask file is designed to exclude inappropriate probe pairs and probe sets, which represent introns or reverse sequences. Average intensity was made equal to target intensity, which was set to 100, to reliably compare variable multiple arrays. In calculating the change of average difference, normalization for all probe sets was performed.

RT-PCR.[3] cDNA was synthesized in 20 μl of reaction volume from 5 μg of total RNA using SuperScript Preamplification System for First Strand cDNA synthesis system (Life Technologies, Inc., Rockville, MD) and oligodeoxythymidylic acid primer and diluted up to 80 μl. PCR was then performed with 1 μl of cDNA for 1 cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s and 68°C for 3 min using the primers listed in Table 1 and Advantage cDNA polymerase mixture (Clontech, Palo Alto, CA). Amplification of the right target DNA was confirmed by mobility on gel electrophoresis and sequencing after subcloning into pGEM-T easy vector (Promega, Madison, WI).

Northern Blot Analysis. Inserts of sequence-verified subclones were amplified by PCR using primers for T7 (5′-TAATACGACTCACTATAGG-3′) and SP6 (5′-GCTATTTAGGTGACA CTATAG-3′) on vector sequence. cDNA fragments for probe were obtained by gel purification of amplified PCR products. Twenty μg of total RNA were denatured and separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde. Equal amount of loading was confirmed by ethidium bromide staining. The gels were blotted to Hybond XL nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ), which were hybridized to [α-^{32}P]dCTP-labeled, random-primed cDNA fragments. Hybridization was performed by using ExpressHyb hybridization solution mixture (Clontech) according to the manufacturer's instructions. The filters were exposed to a BAS imaging plate overnight and visualized by using

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IQGAP1, IQ motif containing GTPase-activating protein 1; SPRR, small proline-rich protein; CRABP2, cellular retinoic acid-binding protein 2; EMP1, epithelial membrane protein 1.

a BAS1800 imaging scanner (Fujifilm, Tokyo, Japan). Signals were quantified by ImageGauge software version 3.1 (Fujifilm).

Results

Global Gene Expression Analysis of Scirrhous Gastric Cancer Cells by High-Density Oligonucleotide Array. We first performed a global analysis of gene expression of 6800 genes in OCUM-2M, OCUM-2MD3, and OCUM-2MLN cells using high-density oligonucleotide array and compared the gene expression profiles of OCUM-2MD3 and OCUM-2MLN with that of OCUM-2M. To eliminate data with low reliability, genes whose expression was regarded as absent in both cell lines by software analysis were excluded. There were 2680 and 2815 remaining genes in OCUM-2MD3 and OCUM-2MLN, respectively. As shown in Fig. 1*A*, OCUM-2MD3 showed broader of differently expressed genes in terms of fold change than OCUM-2MLN. Among the 2680 and 2815 genes expressed, 63 and 23 genes showed a differential expression level greater than 4-fold in OCUM-2MD3 and OCUM-2MLN, respectively. To verify the data, we then performed Northern blot analysis of 100 genes in total, including 86 genes that showed greater than 4-fold difference by oligonucleotide array. When changes greater than 2-fold in expression level by Northern blot analysis are considered significant, consistency with oligonucleotide array analysis was 49% (42 of 86), whereas no significant change was seen in 22% (19 of 86), discordant results were seen in 22% (19 of 86), and undetectable by Northern blot analysis in two cell lines compared in 7% (6 of 86). Genes concordant in both analyses are listed in Table 2, and representative probe tilings after hybridization are shown in Fig. 1*B*. We obtained discordant results with genes that showed elevated expression level in OCUM-2MD3 and OCUM-2MLN by oligonucleotide array; the ratio of concordance was 70% (25 of 36) for down-regulated genes and 34% (17 of 50) for up-regulated genes in OCUM-2MD3 and OCUM-2MLN.

Genes Expressed Differently in OCUM-2MD3 and OCUM-2MLN Compared with OCUM-2M. When gene expression level in OCUM-2M and OCUM-2MD3 was compared, 12 genes showed elevated expression, and 20 genes showed reduced expression in OCUM-2MD3 (Table 2). Up-regulated genes could be classified by function as follows: 4 genes for immunity, 2 genes for trefoil factor, which is a growth factor specific to the digestive tract, and 2 genes for adhesion. On the other hand, down-regulated genes could be classified as follows: 6 genes for differentiation marker of squamous cell lineage, 2 genes for cancer antigen, 2 genes for insulin-like growth factor protein, 1 gene for apoptosis, 1 gene for extracellular matrix, and 1 gene for the candidate tumor suppressor gene. Squamous cell differentiation marker gene SPRR2A, one of the SPRR family genes, was also down-regulated in OCUM-2MD3, as verified only by Northern blot analysis (Fig. 2*A*).

On comparison of OCUM-2M and OCUM-2MLN, five genes showed a high expression level, and five genes showed a low expression level in OCUM-2MLN (Table 2). All five of the up-regulated genes were MHC class II molecules or MHC class II accessory molecules. MHC class II-DMβ was also up-regulated in OCUM-2MLN, as verified only by Northern blot (Fig. 2*B*). Because transcription of MHC class II genes and invariant chain gene is known to be regulated quantitatively by MHC class II transactivator CIITA (14), we next investigated whether expression of CIITA is up-regulated in OCUM-2MLN cells. CIITA was not detectable by Northern blot analysis because of its low expression level, but the expression level of CIITA was relatively higher by RT-PCR in OCUM-2MLN than in OCUM-2M cells (Fig. 3).

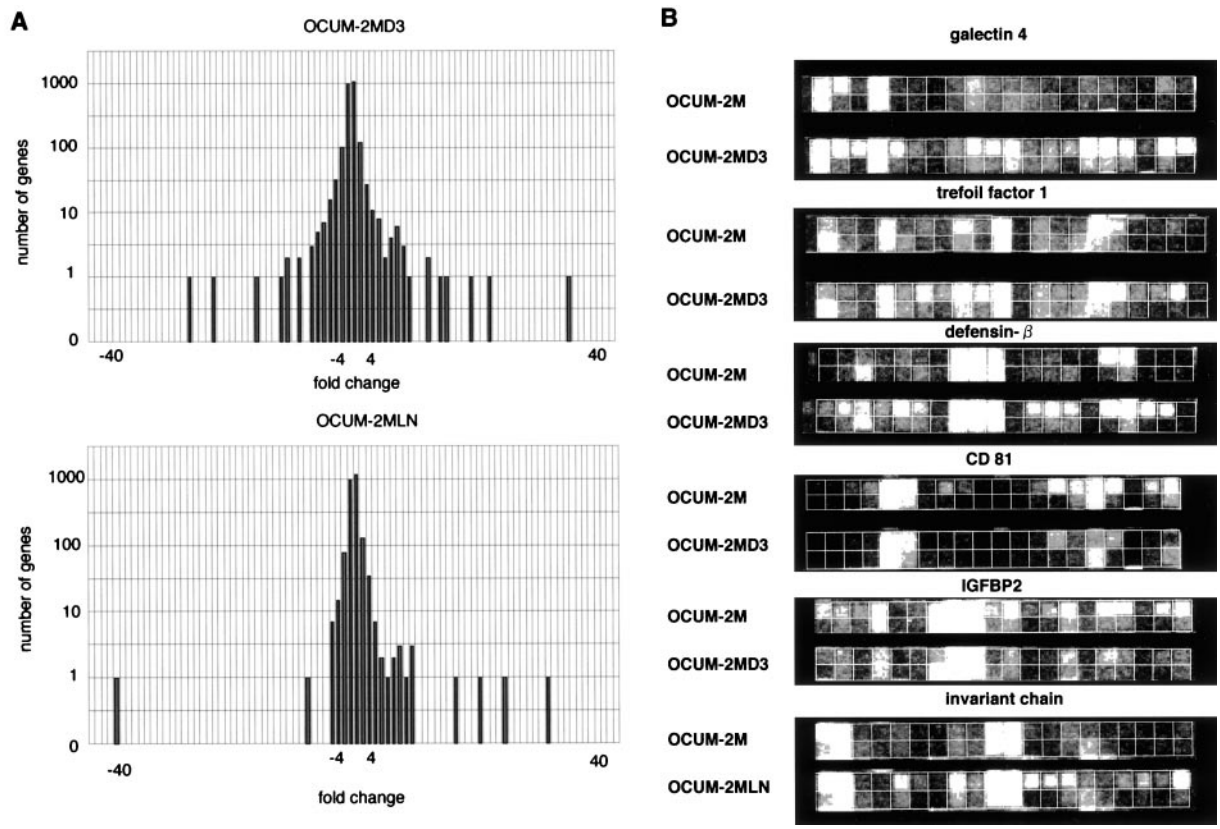

Fig. 1. High-density oligonucleotide array analysis. *A*, histogram analysis of the fold change in gene expression in OCUM-2MD3 and OCUM-2MLN compared with OCUM-2M. Note the number of genes shown in logarithmic scale. *B*, representative probe tilings after hybridization. The *top panels* represent perfectly matched probes, and the *bottom panels* represent corresponding mismatched probes.

Table 2 *Genes that showed differential expression level in OCUM-2MD3 and OCUM-2MLN compared to OCUM-2M*

GenBank accession number, fold change verified by high-density oligonucleotide array and Northern blot, and one representative function of the genes as previously reported are described. Only the genes that showed expression change greater than 4-fold by oligonucleotide array and greater than 2-fold by Northern blot are listed, and genes are sorted by Northern blot data.

No.	GenBank	Northern blot	GeneChip	Gene	Function
OCUM-2MD3					
1	K01396	19.4	6.1	α-1-antitrypsin	Protease inhibitor
2	X52003	16.9	8.3	Trefoil factor 1	Growth factor
3	L08044	8.1	7.7	Trefoil factor 3	Growth factor
4	U59878	7.4	6.7	rab32	Unknown
5	X73079	5.6	8.1	Polymeric-immunogloblin receptor	Immunity
6	AB006781	5.5	14.6	Galectin 4	Adhesion
7	D49824	3.5	12.2	MHC class I, B	Immunity
8	M94880	3.5	4.6	MHC class I, A	Immunity
9	U33053	2.5	4.6	Protein kinase C-like 1	Kinase
10	U30246	2.4	5.9	Na/K/Cl transporter	Transporter
11	U27333	2.1	4	α-1,3-fucosyltransferase	Adhesion
12	Z71389	2.0	35.9	Defensin β2	Immunity
1	M33680	−68.9	−27.6	CD81	Immunity
2	J04152	−49.9	−7.8	GA733-1	Cancer antigen
3	M86934	−29.4	−5.7	GS1	Unknown
4	M97815	−28.7	−5.4	Cellular retinoic acid-binding protein 2	Squamous cell differentiation
5	X82693	−19.6	−6.9	E48	Cancer antigen
6	L05187	−11.0	−4.0	SPRR1A	Squamous cell differentiation
7	X05908	−9.5	−6.8	Annexin A1	Squamous cell differentiation
8	L33075	−9.2	−4.1	IQGAP1	Adhesion
9	S37730	−7.9	−16.1	IGFBP2[a]	Growth and metabolism
10	L27943	−7.6	−4.6	Cytidine deaminase	Nucleic acid metabolism
11	M19888	−6.0	−4.5	SPRR1B	Squamous cell differentiation
12	X93510	−5.5	−12.2	37-kDa LIM domain protein	Candidate tumor suppressor gene
13	M62402	−4.8	−11.7	IGFBP6	Growth and metabolism
14	U40434	−3.6	−11.2	Mesothelin	Squamous cell differentiation
15	M18391	−3.2	−5.8	EphA1	Receptor tyrosine kinase
16	X89986	−3.2	−5.4	Bik	Apoptosis
17	Y07909	−3.2	−4.2	Epithelial membrane protein 1	Squamous cell differentiation
18	M55210	−4.5	−4.5	Laminin γ1	Extracellular matrix
19	U20499	−4.3	−4.3	Sulfotransferase monoamine-preferring	Sulfate conjugation
20	L24774	−2.0	−5.6	Dodecenoyl-CoA δ isomerase	Fatty acid metabolism
OCUM-2MLN					
1	X03100	65.9	4.0	MHC class II DP α	Antigen presentation
2	X00274	13.6	10.7	MHC class II DR α	Antigen presentation
3	M33600	9.3	8.5	MHC class II DR β1	Antigen presentation
4	M13560	5.4	31.9	Invariant chain	Antigen presentation
5	X62744	3.4	7.6	MHC class II DM α	Antigen presentation
1	X53587	−36.4	−4.8	Integrin β_4	Adhesion
2	M58600	−12.0	−4.5	Heparin cofactor II	Protease inhibitor
3	L05187	−11.6	−4.6	SPRR1 A	Squamous cell differentiation
4	M19888	−5.6	−4.7	SPRR1 B	Squamous cell differentiation
5	X65614	−2.0	−41.4	S100 P	Calcium-binding protein

[a] IGFBP, insulin-like growth factor binding protein.

We observed that several genes were down-regulated in both OCUM-2MD3 cells and OCUM-2MLN cells. In addition to SPRR1A and SPRR1B, squamous cell differentiation markers such as SPRR2A, annexin A1, and EMP1 also showed reduced expression in OCUM-2MLN cells, although to a lesser extent than that seen in OCUM-2MD3 cells (Fig. 2*A*). Integrin β_4 and E-cadherin, which play crucial roles in peritoneal dissemination and invasion of gastric cancer, respectively, were also down-regulated in both cell lines, as verified by Northern blot analysis (Fig. 4).

Expression of Selected Genes in Nine Gastric Cancer Cell Lines. Because several reports have previously described peritoneal dissemination potentials of other gastric cancer cell lines, we also investigated the expression of the genes differentially expressed between OCUM-2M and OCUM-2MD3 by Northern blot on another six gastric cancer cell lines, in addition to the three cell lines analyzed here, to find any correlation between these genes and the peritoneal dissemination potential of gastric cancer cells (Fig. 4). MKN45 and MKN74 have been widely used as a peritoneal dissemination model in nude mice (6, 15), with a higher potential for peritoneal dissemination reported in MKN45 (7). Ishii *et al.* (5) reported that after injection of 5×10^6 cells of MKN7, MKN74, and MKN45 into the peritoneal cavity of severe combined immunodeficient mice, 2.3, 41.8, and 149.5 peritoneal nodules were formed, respectively. NUGC-4 is reported to induce peritoneal metastasis, leading to severe carcinomatous peritonitis, whereas NUGC-3 did not form a peritoneal metastasis even at 4 weeks after inoculation (16). No colony of peritoneal dissemination was observed when 1×10^7 OCUM-2MLN cells were injected i.p.[4]

Taken together, these cells can be readily classified into two groups: (*a*) cells with a high potential for peritoneal dissemination (OCUM-2MD3, MKN45, MKN74, and NUGC-4); and (*b*) cells with low potential or no potential for peritoneal dissemination (OCUM-2M, OCUM-2MLN, MKN7, and NUGC-3). Although there are no reports on the peritoneal dissemination potential of GT3TKB in the nude mice system, we included it in our study because it is one of the cell lines derived from disseminated nodules on the peritoneum. Trefoil factor 1, α-1-antitrypsin, and galectin 4 showed a high expression level only in cells with a high potential for peritoneal dissemination and a low expression level in all of the cells with a low potential for peritoneal dissemination. On the contrary, cytidine deaminase showed a reciprocal expression pattern. The expression

[4] M. Yashiro, unpublished data.

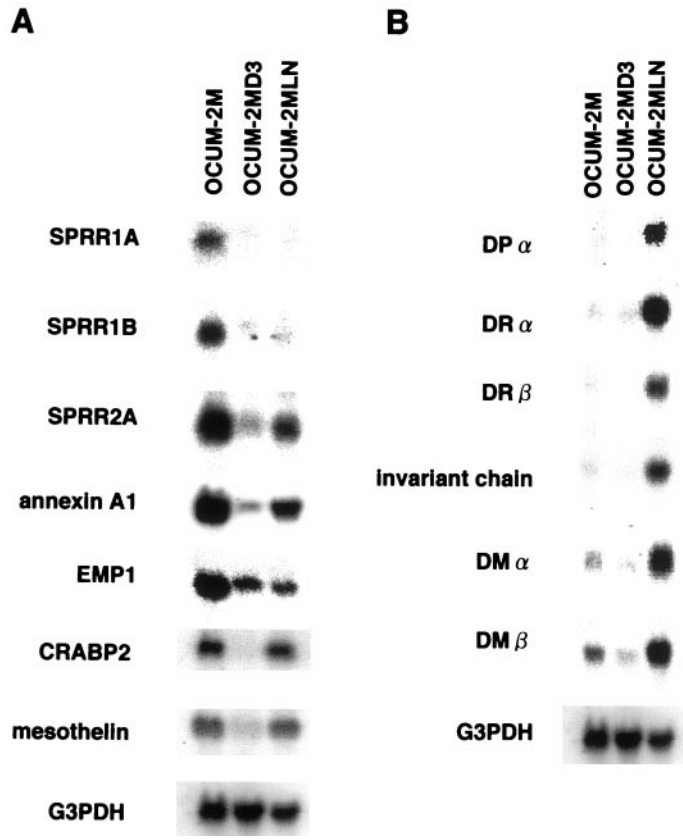

Fig. 2. Northern blot analysis of two gene clusters. OCUM-2M, OCUM-2MD3, and OCUM-2MLN were analyzed. *A*, genes related to differentiation of squamous cells were coordinately down-regulated in OCUM-2MD3. *B*, genes involved in antigen presentation were coordinately up-regulated in OCUM-2MLN.

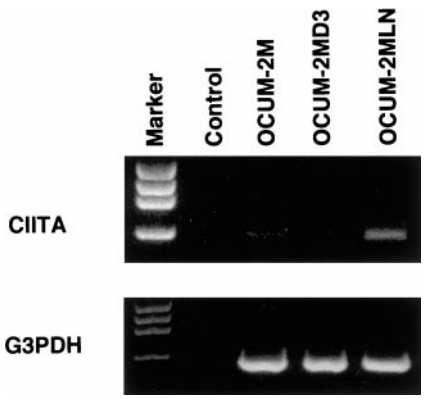

Fig. 3. RT-PCR analysis of CIITA. OCUM-2M, OCUM-2MD3, and OCUM-2MLN were analyzed. The highest level of expression is detected in OCUM-2MLN. G3PDH was shown as an internal control. *Control* and *Marker* represent the negative control and ϕX174–*Hae*III, respectively.

pattern of integrin β_4 and E-cadherin did not show any correlation to peritoneal dissemination potential.

Discussion

With regard to peritoneal dissemination, malignant characteristics of cancer cells such as decreased intercellular adhesion, increased cell to matrix adhesion, and resistance to apoptosis have been considered to be important. Several of the 32 genes that showed different expression levels in OCUM-2M and OCUM-2MD3 in this study have been reported previously to have a relationship with these features. For example, α-1,3-fucosyltransferase, which is essential for sialyl-Le(X) and sialyl-Le(A) antigen, is reported to be expressed in the peritoneum, to facilitate the attachment of cancer cells to the peritoneum, and to promote liver metastasis of some cancers via E-selectin-mediated adhesion (7, 17). Trefoil factors 1 and 3, growth factors of the gastrointestinal tract that play pivotal roles in restitution after mucosal injury, are reported to promote cell growth and motility as well as down-regulate E-cadherin expression (18, 19). As a result, autocrine production of these growth factors by cancer cells could be highly advantageous to invasion and metastasis. Bik induces apoptosis by interacting with Bcl-2 (20). Decreased expression of Bik might prolong cell survival, thereby promoting peritoneal dissemination because overexpression of BAG-1, a Bcl-2 family gene, can lead to enhanced peritoneal dissemination of gastric cancer through an anti-apoptotic effect (6). Without laminin γ1, which makes a heterotrimer with other α and β subunits, mature laminin is not produced, leading to a complete absence of the basement membrane (21). It is reported that decreased expression of laminin, as often seen in various gastric cancer cell lines, causes basement membrane fragility (22). Thus, reduced expression of laminin γ1 might facilitate tumor invasion by concomitant incomplete barrier formation. IQGAP1, a negative regulator of E-cadherin-mediated cell to cell adhesion (23), was down-regulated. A recent study (24) reported that gastric hyperplasia are observed in the IQGAP1(−/−) mouse, suggesting another function of

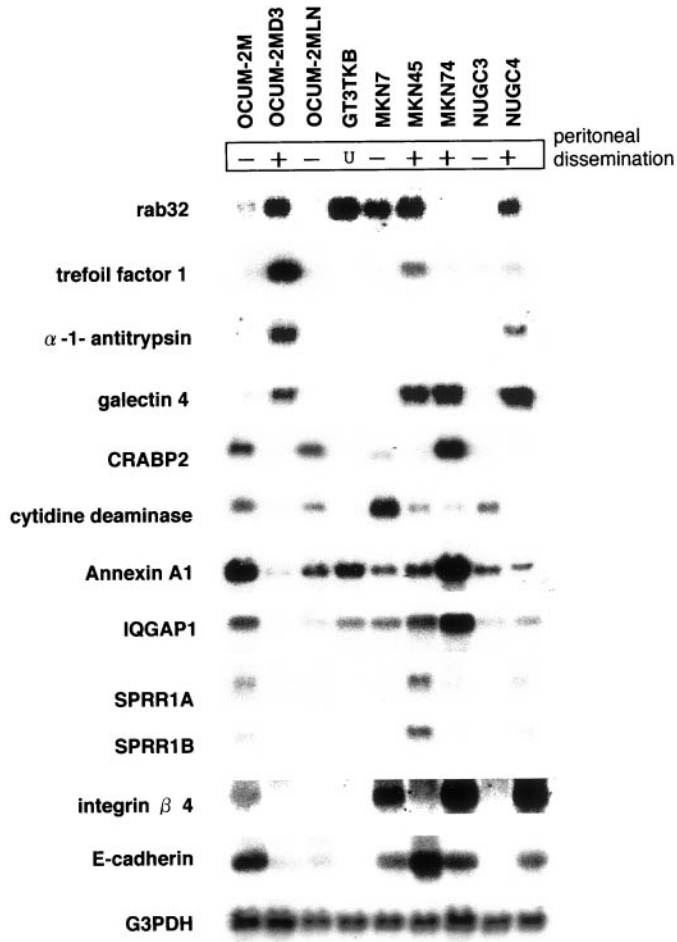

Fig. 4. Representative Northern blot analysis of differentially expressed genes. Nine gastric cancer cell lines were analyzed. The potential for peritoneal dissemination in a nude mice model as reported elsewhere was also described by + (high potential), − (very low or no potential), or *U* (unknown potential). G3PDH was shown as an internal control.

IQGAP1 in relation to overgrowth. In this context, reduced expression of IQGAP1 might involve cell proliferation rather than reduced negative regulation of cell to cell adhesion.

Down-regulation of the squamous cell differentiation marker gene cluster in OCUM-2MD3 was obvious. Squamous cell differentiation marker included cornified envelope-related genes, CRABP2, and mesothelin. SPRR1A, SPRR1B, SPRR2A, and annexin A1 are all major constituents (25), and EMP1 was reported to be coexpressed with these four genes (26). Cornified envelope is observed only in squamous cells such as differentiated keratinocytes, but we could detect expression of one of the cornified envelope-related genes, SPRR1B, in adenocarcinoma cell lines that originated from the breast, pancreas, and colon (data not shown), therefore suggesting possible different roles for this gene cluster other than cornification. Interestingly, these cornified envelope-related genes were also down-regulated in OCUM-2MLN. Thus, reduction of cornification-related genes might have a function that is relevant in metastasis. Coordinate decreased expression of these functionally related genes implicates an upstream regulator; one possible regulator is retinoid because cornified envelope genes are reported to be suppressed by retinoic acid (27), and CRABP2 mediates retinoic acid signal transduction (28). Another cell surface marker, mesothelin, which is literally a differentiation marker of mesothelium that covers the peritoneum, regulates the traffic of molecules and cells in and out of the peritoneal cavity (29). It is probable that reduced expression of mesothelin can alter the adhesiveness of cancer cells to the peritoneum, although there is limited information on the matter of this gene function.

On the other hand, in gastric cancer cell lines with a high potential for peritoneal dissemination such as MKN45, MKN74, NUGC-4, and OCUM-2MD3, there was a tendency for galectin 4, α-1-antitrypsin, and trefoil factor 1 to show a high expression level and for cytidine deaminase to show a low expression level. Whether these genes are involved in peritoneal dissemination is a matter to be further investigated.

Up-regulation of MHC class II genes and invariant chain gene in OCUM-2MLN was extremely intriguing. Expression of these antigen gene clusters is often observed in a solid tumor, although its significance to cancer cells is unclear. It was reported that on the surface of OCUM-2M and OCUM-2MLN cells, MHC class II antigens were not detected by flow cytometry analysis at all (9), suggesting that these molecules may not be functionally intact or stable. Transcription of MHC class II genes and invariant chain gene has been considered to be regulated by MHC class II transactivator CIITA constitutively in B cells or dendritic cells and inducibly by IFN-γ in nonlymphoid tissues (30, 31). In addition, the expression level of CIITA was reported to be proportional to that of MHC class II genes (14). In this study, a higher expression level of CIITA was seen by RT-PCR in OCUM-2MLN as compared with OCUM-2M, as predicted. When analyzed by oligonucleotide array, differential expression of CIITA between both cell lines was not reliably detected, presumably because of a subtle change within a low expression level. However, we could finally detect the difference by deducing an upstream regulator gene from the expression profile analysis. We believe that this is also an effective way to make use of array technology and that the sensitivity of array technology must be improved in the future. E-cadherin and integrin β_4, genes relevant for gastric cancer metastasis, were down-regulated in both of the metastatic sublines of OCUM-2M. E-cadherin is regarded as an invasion suppressor in gastric cancer and other types of cancer (3), and integrin β_4 has recently been reported as a suppressor of peritoneal dissemination of gastric cancer (5). Suppressed expression of these genes in the highly metastatic cells, as we have shown in this study, is in good concordance with previous reports and indicates that gene expression change in these cells properly reflects some metastatic characteristics. In this study, however, these genes showed various expression patterns among nine gastric cancer cell lines, and no apparent correlation was observed with peritoneal dissemination potential. These results clearly show that a complex phenomenon *in vivo* such as peritoneal dissemination cannot be explained by altered expression of a single gene. Thus, the relevance of exploring the global gene expression profile by using a comprehensive procedure is obvious.

Although the initial discrepancy between the data by high-density oligonucleotide array and the data by Northern blot was not negligible in this study, the final data presented here were verified by two different methods and must be highly reliable. Oligonucleotide array analysis greatly facilitated clarification of a whole aspect of gastric cancer metastasis by showing the global changes in gene expression and revealing two differently regulated gene clusters, which have not been reported in relation to metastasis. Based on this study, further investigations are now required for all of the genes discussed above to verify their involvement in peritoneal dissemination and lymph node metastasis.

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