Identification of genes differentially expressed in a newly isolated human metastasizing esophageal cancer cell line, T.Tn-AT1, by cDNA microarray

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We isolated a metastasizing human esophageal squamous cell carcinoma (SCC) cell line, T.Tn-AT1, from a parental non-metastasizing cell line, T.Tn, by *in vitro* selection and by use of a nude mouse orthotopic inoculation model. Then, we compared the expression profiles of 9206 genes in T.Tn-AT1 and T.Tn by cDNA microarray analysis. The gene expression profiles of T.Tn and T.Tn-AT1 were very similar, and only 34 genes showed more than 3-fold differential expression. Among the 34 genes, 29 genes were down-regulated and only 5 genes were up-regulated in T.Tn-AT1 cells. Subsequently, we confirmed the expression levels of 14 of the 34 genes in T.Tn and T.Tn-AT1 cells by means of reverse transcription-polymerase chain reaction. The expression of 8 genes (*KAL1, HPGD, NDN, REG1A, CXCR4, SPOCK, DIAPH2 and AIF1*) was down-regulated and that of one gene (*VNN2*) was up-regulated in T.Tn-AT1 cells. These 9 genes encoded proteins associated with metastatic processes, such as adhesion, migration, inflammation, proliferation, and differentiation. Thus, these genes might regulate the metastasis of esophageal SCC, and could be predictive markers for lymph node metastasis of esophageal SCC. (Cancer Sci 2003; 94: 699–706)

Esophageal squamous cell carcinoma (SCC) is one of the most aggressive cancers. Esophageal SCC is relatively common in countries in Eastern Asia, such as China and Japan, and is characterized by poor prognosis and rapid clinical progression, with a high frequency of lymph node metastasis and recurrence. The 5-year survival rate of patients with esophageal SCC showing submucosal invasion is low (40–75%)[1–5] compared with that for patients with colon cancer (over 80%).[4,5] In addition, lymph node metastasis is commonly found in esophageal SCC, even when the tumor invades only the submucosa. Lymph node metastasis is the main cause of the poor prognosis for the patients with esophageal SCC.

Consequently, the identification of the genes associated with metastasis of esophageal SCC is very important. Microarray analysis has been used to investigate the gene expression profiles of esophageal SCC tissues and esophageal cancer cell lines with various characteristics.[6–10] When clinical materials are used for microarray analysis, it might be necessary to look at the overall expression profiles of genes (if possible) by clustering analysis in the different pathological stages, such as dysplasia, carcinoma *in situ*, and invasive cancer with or without metastasis. However, to isolate gene(s) related to metastasis, it would be convenient to use cell lines with different metastatic potentials derived from the same parental cells, and a reliable model system is therefore required for examining the metastatic potential of cancer cells.

In our previous experiments, we established *in vitro* and *in vivo* model systems for studying invasion and metastasis of esophageal SCC cells.[11,12] We first clarified in detail the molecular and genetic characteristics of a human non-metastasizing esophageal SCC cell line, T.Tn,[12] and then developed an orthotopic inoculation model for esophageal cancer cells in nude mice.[11] In the present study, we isolated a metastasizing subclone from the parental non-metastasizing T.Tn cell line by *in vitro* selection and by the use of a nude mouse orthotopic inoculation model. Then, we compared the expression profiles of 9206 genes in the parental T.Tn cells and the metastasizing subclone by cDNA microarray analysis, and identified several genes differentially expressed in the metastasizing subclone.

Materials and Methods

Cell line. A human esophageal SCC cell line, T.Tn[13] was obtained from JCRB (Japanese Collection of Research Bioresources, Osaka). T.Tn cells were grown in 1:1 mixture of Dulbecco's modified Eagle's medium (Nissui, Tokyo) and F-12 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO), 100 μg/ml streptomycin, 100 units/ml penicillin (Life Technologies, Inc.), and 0.25 μg/ml amphotericin B (Life Technologies, Inc.) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. HT1080 cells are derived from a human fibrosarcoma cell line known to secrete a large amount of several matrix-degrading enzymes (purchased from Dai-Nippon Seiyaku, Osaka). HT1080 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS and antibiotics.

Isolation of subclones with high migrating ability *in vitro*. In order to isolate subclones with high migrating ability from T.Tn cells, we used Transwell Invasion Chambers (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). T.Tn cells (7.5×10^4) were seeded on the upper compartment of the invasion chamber, which had a polycarbonate filter with 8-μm pore size. After 48-h incubation, the upper compartment was removed from the chamber, and the cells which had migrated into the lower compartment were cultured until they reached confluency. Then, these cells were re-inoculated into the upper compartment of a new invasion chamber, and the same selection procedure was repeated five times. Cells with high migrating ability on the lower chamber after final selection were sub-cultured in a 100-mm dish (Falcon; Becton Dickinson Labware), and several subclones were isolated by a limiting dilution technique as described previously.[14]

Isolation of subclones with high cell-substrate and cell-cell adhesive ability *in vitro*. T.Tn cell suspension (3×10^5) was inoculated into 6-well plates (Falcon; Becton Dickinson Labware) which had been coated with type I collagen (2.5 mg/cm^2; Falcon;

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Becton Dickinson Labware). Immediately after inoculation, the plate was rotated at 60 rpm for 10 min, and the medium containing non-attached cells was removed. Then the plate was gently washed with the medium without FCS twice, and the medium with FCS was added to the plate. After 10 days of incubation, a few colonies were formed in the plate. The cells were harvested by treatment with trypsin (0.05%) and EDTA (0.53 m*M*) for 5 min and the single cell suspension was inoculated again into a collagen-coated plate. The plate was rotated at 60 rpm for 2 min at this time, and the same selection procedure was repeated three times. We thus obtained cells which showed high adhesive ability to the substrate (type I collagen).

The cells with high adhesive ability to type I collagen were seeded on 100-mm plastic dishes. After they had reached subconfluency, the cells were treated with trypsin (0.05%) and EDTA (0.53 m*M*) for 20 min, and the detached cells were removed by washing with the medium without FCS twice. Then, the cells which showed strong cell-cell adhesion and remained attached on the plate as a cell-cluster were cultured until subconfluency. The same selection was repeated three times to afford several subclones with high cell-substrate and cell-cell adhesive ability.

***In vivo* tumorigenicity and metastasis assay.** Six-week-old male BALB/c nude mice were obtained from Clea Japan, Inc., Tokyo. The mice were kept in sterilized cages equipped with an air filter and sterile bedding materials, and were given sterilized water and food throughout the study period. The tumorigenicity, invasiveness and metastatic potential of the cancer cells were examined in the nude mice. Cells (1×10^7) were suspended in 0.05 ml of serum-free medium, and injected into the s.c. tissue. The mice received s.c. tissue injection were killed at 150 days after inoculation. At autopsy, the s.c. tumors, lymph nodes (axillary and inguinal), lungs, liver, spleen and kidney were inspected for gross tumors. After 24-h fixation of the tissues in 10% neutral buffered formalin at room temperature, representative sections of these organs were processed for routine histological examination.

Orthotopic inoculation of cancer cells. Orthotopic inoculation of the esophageal cancer cells was carried out according to the reported method.[11] The mice receiving cancer cells were killed at 6 or 8 weeks after inoculation. At autopsy, the esophagus, stomach, lungs, liver, spleen, kidney, peritoneum and lymph nodes (mediastinal and intra-abdominal) were inspected for gross tumors, and representative sections of these organs were processed for routine histologic examination.

This experiment was carried out under the control of the Animal Care and Use Committee, Dokkyo University School of Medicine, in accordance with Guidelines for the Care and Use of Laboratory Animals, Dokkyo University School of Medicine.

Gelatin zymography and reverse gelatin zymography. Cells were grown to confluence in the serum-supplemented medium, washed twice with serum-free medium, and cultured for an additional 48-h in serum-free medium. The medium was collected, centrifuged at 3800 rpm, and concentrated approximately 200-fold using an Ultrafree-15 Centrifugal Filter Device (Millipore, Bedford, MA). The protein concentration in the samples was determined by means of the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). SDS-substrate polyacrylamide gel electrophoresis was performed as described previously.[15] Gels for zymography were composed of gelatin (0.1% w/v; Sigma) and polyacrylamide (10% w/v), and for reverse zymography, of gelatin (0.1% w/v), polyacrylamide (12.5% w/v), and concentrated conditioned medium (100 μg/ml) of HT1080 as a source of gelatinases. Protein samples were added to a loading buffer [50 m*M* Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, and 10% glycerol] and were not heated before electrophoresis. Gels were run at 30 mA/gel at 4°C, incubated overnight at 37°C in 50 m*M* Tris-HCl buffer (pH 7.4) containing 10 m*M* $CaCl_2$, stained with Coomassie blue (0.25% w/v), and destained in methanol:acetic acid:water solution (45:10:45). Clear zones indicated the presence of gelatinolytic activity in zymography, while in reverse zymography, dark zones represented the activity of gelatinase inhibitors.[16]

Western blotting. Cells grown to sub-confluence were washed with D-PBS without Ca^{2+} and Mg^{2+} twice, and the cells were lysed in 20 m*M* Tris-HCl buffer (pH 7.4) containing 150 m*M* NaCl, 2% NP-40 (Sigma), 5 m*M* EDTA, 2 m*M* NaN_3, 0.1 m*M* phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 20 μg/ml aprotinin. The protein concentration of the samples was determined by Bio-Rad Protein Assay. Fifty-microgram protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to nitrocellulose (Bio-Rad), and E-cadherin and β-catenin were detected on the nitrocellulose with an anti-E-cadherin mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) or an anti-β-catenin mouse monoclonal antibody (Transduction Laboratories), and with an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

Microarray analysis. Cancer cells grown in monolayers were harvested at early confluence. Cytoplasmic RNA was prepared with ISOGEN RNA extracting mixture (Nippon Gene, Toyama). RNA integrity was confirmed by visualizing intact 28S and 18S ribosomal RNAs on formaldehyde denaturing aga-

Table 1. Primers used

Gene name (definition)	Upstream	Downstream	Length of DNA fragment (bp)	Cycles
AB033010	gctaccctaaaggccactcg	tgggaagcaccttcactcc	63	28
AK001803	ctgctgtgtggctcgatatga	gatggttgccaaaacctatgc	68	28
NM_000216 (KAL1)	gcgaagatgacgccactca	cagtcagttgaactcgctcgtct	66	28
NM_000860 (HPGD)	tgagtaagcaaaatggaggtgaag	gctgtgcaacgggcatg	78	28
NM_002487 (NDN)	gtaaggatctgagcgaccctaact	gctgctgtgcacctcgg	62	32
NM_002909 (REG1A)	cagatctctattgccagaacatgaa	accctcggcctgggtg	65	32
NM_003467 (CXCR4)	cagcggttaccatggaggg	ttccttcatggagtcatagtccc	89	32
NM_003708 (RODH-4)	gctgcagccgccca	attcaccaggccccagagtc	63	32
NM_004598 (SPOCK)	ctctccacgaggatgcgaa	tgctagtgtcaaacctgccttg	75	28
NM_004665 (VNN2)	ggaggatgccttgaatctcatg	gattcgagcaccctgctcag	85	40
NM_006729 (DIAPH2)	caatccggtcagctgggtta	aagcttttccagctcatccaat	73	28
NM_006891 (CRYGD)	gctcctgccgcctcatc	gcctctgtagtcctccctcta	71	32
NM_012331 (MSRA)	ccgggccggaagga	cgactgttctgttgccattga	64	28
NM_032955 (AIF1)	ggagacgttcagctaccctga	caggatcatttttaggatggcag	73	40
M33197 (GAPDH)	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	226	20

Table 2. Tumorigenicity and metastatic potential of T.Tn cell line and its subclones in nude mice after s.c. inoculation

Clone	Tumorigenicity	Tumor volume	Metastases		
			Lymph nodes	Lungs	Other organs
T.Tn	11/12[1)] (n=4)	42.6±17.5[2)]	0/4[3)]	0/4[3)]	0/4[3)]
T.Tn-Mgr1	10/11 (n=4)	32.7±17.5	0/4	0/4	0/4
T.Tn-AT1	9/9 (n=3)	193.6±52.4[4)]	0/3	2/3	0/3

1) Number of tumors/inoculated sites.
2) Mean±SEM mm³.
3) Number of mice with metastasis/number of mice with tumor.
4) Statistically significant when compared to those of T.Tn and T.Tn-Mgr1 (one-way ANOVA, P<0.02).

rose gel. Microarray analysis was performed (Kurabo, Osaka) on a "CodeLink" Bioarray ("Motorola," Anaheim, CA) containing 9206 spots of 30-mer oligonucleotides.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The RNA from T.Tn and T.Tn-AT1 cells was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) at 42°C for 60 min using random primer (5 μ*M*; Life Technologies, Inc.) in 20 μ1 of the reaction mixture. Subsequently, 1 μl of the products was subjected to PCR amplification. PCR was performed as follows: the final concentrations of dNTPs and primers in the reaction mixture were 200 μ*M* and 1 μ*M*, respectively. *Taq* DNA polymerase (TaKaRa Biomedicals, Kusatsu) was added to the mixture at a final concentration of 0.05 unit/μ1, and the reaction was carried out in a TaKaRa Thermal Cycler MP (TaKaRa Biomedicals) under the following conditions: 94°C for 3 min and then 94°C for 1 min, 62°C for 1.5 min, 72°C for 2.5 min for an appropriate number of cycles for each gene (Table 1), and extension at 72°C for 4 min.

To determine whether conditions were adequate for semi-quantitative RT-PCR, we amplified the fragments under the same PCR conditions with different numbers of cycles (16, 20, 24, 28, 32, 36 and 40 cycles). Primers were designed by "Primer Express" (Applied Biosystems, Foster City, CA), and are listed in Table 1.

Statistical analysis. Statistical analysis was performed with a one-way ANOVA, and P<0.05 was considered significant.

Results

Isolation of subclones with high migrating ability or with high cell-substrate and cell-cell adhesive ability. Several subclones with high migrating ability or with high cell-substrate and cell-cell adhesive ability were isolated from parental T.Tn cells. We examined the growth profile *in vitro* and the tumorigenicity in nude mice after s.c. administration of all subclones, and selected a representative subclone from each group for further study. A subclone with high migrating ability was designated as T.Tn-Mgr1, and another subclone with high cell-substrate and cell-cell adhesive ability was designated as T.Tn-AT1. *In vitro* growth potential of T.Tn-Mgr1 and T.Tn-AT1 was similar to that of parental T.Tn cells (data not shown).

***In vivo* tumorigenicity and metastatic potential of T.Tn, T.Tn-Mgr1 and T.Tn-AT1.** When we inoculated parental T.Tn cells and the subclones T.Tn-Mgr1 and T.Tn-AT1 into s.c. tissue of nude mice, all the mice developed tumors (Table 2). The mean volume of tumors formed by T.Tn-AT1 was significantly larger than that formed by the parental T.Tn cells and T.Tn-Mgr1 (P<0.02, one-way ANOVA). Tumors formed by T.Tn (Fig. 1A) and T.Tn-Mgr1 (data not shown) were well-differentiated SCC, but tumors formed by T.Tn-AT1 were poorly differentiated SCC (Fig. 1B). T.Tn tumor (Fig. 1A) and T.Tn-Mgr1 tumor (data not shown) were minimally invasive into the muscle and the skin, whereas T.Tn-AT1 tumor (Fig. 1B) was massively invasive into the muscle and vertebra. T.Tn tumor and T.Tn-Mgr1 tumor did not metastasize to regional lymph nodes or to any

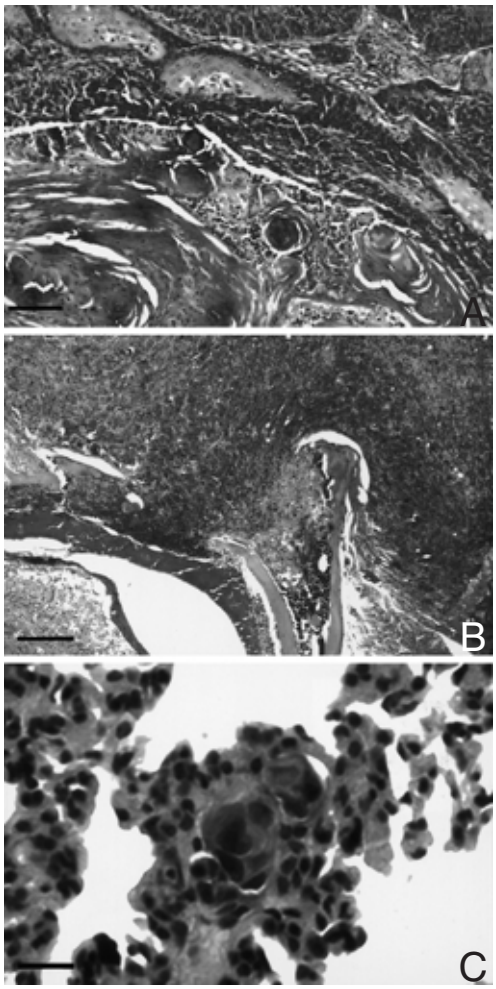

Fig. 1. Histopathological appearance of T.Tn and T.Tn-AT1 tumors in nude mouse s.c. (A) The tumors formed by T.Tn were well-differentiated SCC, minimally invasive into the muscle and the skin. (B) The tumors formed by T.Tn-AT1 were poorly differentiated SCC, and were massively invasive into the muscle and vertebral bone. (C) T.Tn-AT1 tumor developed tumor emboli in the alveolar capillaries (pulmonary involvement). Scale bar=120 μm (A), 300 μm (B), 30 μm (C).

Table 3. Tumorigenicity and metastatic potential of T.Tn cell line and its subclone T.Tn-AT1 at the orthotopic site (esophagus) in nude mice

Clone	Tumorigenicity at the orthotopic site	Metastases		
		Lymph nodes	Lungs	Other organs
T.Tn	1/6[1)]	0/1[2)]	0/1	0/1
T.Tn-AT1				
6 weeks	3/5	2/3	0/3	0/3
8 weeks	2/4	1/2	0/2	0/2

1) Number of mice with tumor/number of mice receiving cancer cells.
2) Number of mice with metastasis/number of mice with tumor at esophagus.

distant organ, but T.Tn-AT1 tumor developed tumor emboli in the alveolar capillaries (pulmonary involvement) (Fig. 1C).

Orthotopic inoculation of T.Tn and T.Tn-AT1. T.Tn cells formed poorly differentiated SCC in the submucosal layer of the esophagus (data not shown). There was no microscopic evidence of metastases to the lymph nodes or any distant organ (Table 3). The mice receiving T.Tn-AT1 cells developed bulky tumors at the esophagus. T.Tn-AT1 tumor was poorly differentiated SCC, and massively invasive into esophageal wall (Fig. 2A). T.Tn-AT1 tumor showed lymphatic and venous invasion (Fig. 2B), and peritoneal dissemination (Fig. 2C). Furthermore, T.Tn-AT1 tumor metastasized to regional lymph nodes (Table 3). However, distant metastases by T.Tn-AT1 tumors were not detectable during the observation period (Table 3).

Because of technical difficulty in the orthotopic inoculation of the esophageal cancer cells, only limited tumor formation (5 tumors formed per 9 inoculated mice) and lymph node metastasis (3 metastases per 5 tumors) by T.Tn-AT1 cells were obtained in our orthotopic inoculation model. However, because the parental T.Tn cells rarely formed tumors at the inoculated site (1 tumor formed per 6 inoculated mice) and never metastasized to the lymph node in the same model operated by the same person (T. F.), we concluded that T.Tn-AT1 cells had high metastatic potential in nude mice, and used T.Tn-AT1 cells as metastasizing esophageal cancer cells for further experiments. Two years after subcloning of the T.Tn-AT1 cells, the metastatic potentials of the cells in nude mice have remained stable (data not shown).

Production of matrix-degrading enzymes and their inhibitors by T.Tn cells and its subclones. Zymographic analysis showed that the parental T.Tn cells secreted 20, 68, 80 and 92 kDa gelatinolytic enzymes (Fig. 3A). However, the expression levels of the gelatinases in the parental T.Tn cells were much lower than those in HT1080 cells. The gelatinases of 68, 80 and 92 kDa are considered to be proMMP2, active MMP9 and proMMP9 respectively. The faint band of 20 kDa gelatinase is considered to be proMMP7. The expression level of proMMP2 in T.Tn-Mgr1 cells is higher than that in the parental T.Tn cells. However, the expression level of proMMP2 in T.Tn-AT1 was lower than that in the parental T.Tn cells. The expression levels of proMMP9 and active MMP9 in T.Tn-Mgr1 and T.Tn-AT1 were lower than those in the parental T.Tn cells.

T.Tn cells secreted 21 kDa and 31 kDa gelatinase inhibitors, as detected by reverse gelatin zymography (Fig. 3B). The gelatinase inhibitor of 31 kDa was considered to be TIMP1, and that of 21 kDa was considered to be TIMP2. The levels of TIMP activities in T.Tn-Mgr1 and T.Tn-AT1 were similar to those in the parental T.Tn cells.

Expression of β-catenin and E-cadherin in T.Tn cells and the subclones. We previously reported that T.Tn cells expressed moderate levels of E-cadherin and β-catenin.[12)] The expression levels of E-cadherin and β-catenin proteins in T.Tn-Mgr1 and T.Tn-AT1 were similar to those in the parental T.Tn cells (Fig. 4, A and B).

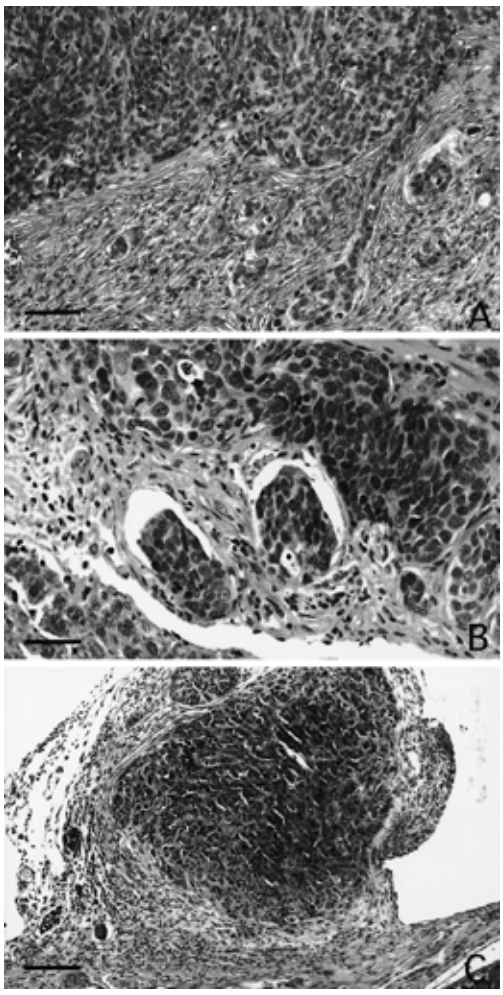

Fig. 2. Orthotopic inoculation of T.Tn-AT1. (A) T.Tn-AT1 cells formed bulky tumors at the esophagus. T.Tn-AT1 tumors were poorly differentiated SCC, massively invasive into the esophageal wall. (B) T.Tn-AT1 tumors showed lymphatic and venous invasion, and (C) peritoneal dissemination. Scale bar=60 μm (A), 45 μm (B), 300 μm (C).

Microarray analysis. As we expected, the gene expression profiles of T.Tn cells and T.Tn-AT1 cells were very similar (Fig. 5). When we looked for genes that were more than 3-fold differentially expressed in T.Tn and T.Tn-AT1 cells, 34 genes out of 9206 genes were picked up by microarray analysis (Table 4). Twenty-nine of the 34 genes were down-regulated in the metastasizing T.Tn-AT1 cells, and 5 were up-regulated. Although T.Tn-AT1 cells were isolated on the basis of high adhesive ability to type 1 collagen and high cell-cell adhesion, there was no up-regulation of the integrin family (β2, β3, β4, β5, β6, β7, β8

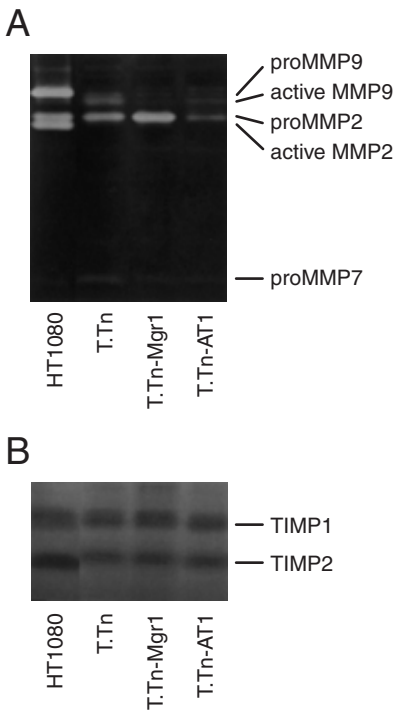

Fig. 3. (A) Gelatin zymography and (B) reverse gelatin zymography of conditioned medium. Gels for zymography were composed of gelatin (0.1% w/v) and polyacrylamide (10% w/v), and for reverse zymography, of gelatin (0.1% w/v), polyacrylamide (12.5% w/v), and concentrated conditioned medium (100 μg/ml) of HT1080 as a source of gelatinases. Protein samples (for zymography, 1 μg from HT1080, 20 μg from T.Tn and its subclones; for reverse zymography 20 μg from all cells) were added to a loading buffer [50 m*M* Tris-HCl (pH 7.4), 2% SDS, 0.1% bromophenol blue, and 10% glycerol] and were not heated before electrophoresis. Gels were run at 30 mA/gel at 4°C, incubated overnight at 37°C in 50 m*M* Tris-HCl buffer (pH 7.4) containing 10 m*M* $CaCl_2$, stained with Coomassie blue (0.25% w/v), and destained in methanol:acetic acid:water solution (45:10:45). Clear zones indicate the presence of gelatinolytic activity in zymography, while in reverse zymography, dark zones represent the activity of gelatinase inhibitors.

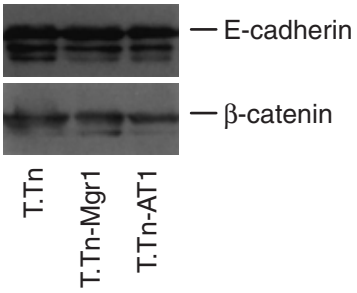

Fig. 4. Western blot analysis of β-catenin and E-cadherin in T.Tn cells and the subclones. Fifty-microgram protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to nitrocellulose, and E-cadherin and β-catenin were detected on the nitrocellulose with an anti-E-cadherin mouse monoclonal antibody or an anti-β-catenin mouse monoclonal antibody, and with an ECL kit.

and α2, α3, α4, α5, α6, α7, α9, α10) in T.Tn-AT1 cells compared to T.Tn cells as judged from this microarray analysis (data not shown).

Differential expression of the genes in T.Tn and T.Tn.-AT1. We selected 14 genes (Table 1) among the 34 differentially expressed genes, as being potentially important for acquisition of metastatic potential by carefully examining the original expression

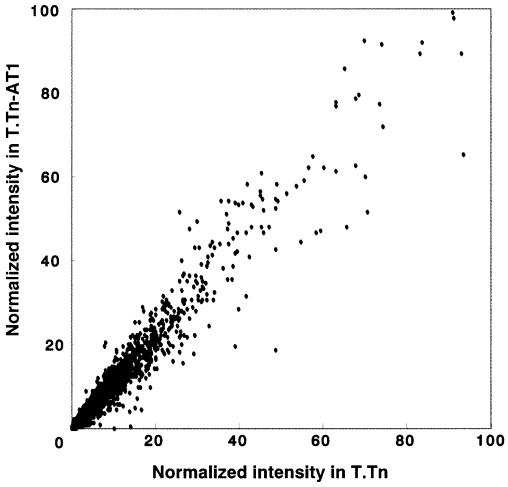

Fig. 5. Gene expression profile of T.Tn and T.Tn-AT1 cells. Microarray analysis was performed on the "CodeLink" Bioarray containing 9206 spots of 30-mer oligonucleotides.

levels and altered expression levels, and by reviewing the literature concerning these genes. Then we examined the expression levels of the 14 genes in T.Tn and T.Tn-AT1 cells by semi-quantitative RT-PCR. Nine genes (*KAL1, HPGD, NDN, REG1A, CXCR4, SPOCK, VNN2, DIAPH2* and *AIF1*) among the 14 genes were found to be differentially expressed between T.Tn and T.Tn-AT1 cells (Fig. 6). The differential expression patterns of all 9 genes were consistent with the results obtained from microarray analysis. However, the expression levels of 5 genes (*AB033010, AK001803, RODH-4, CRYGD* and *MSRA*) were found to be similar in T.Tn and T.Tn-AT1 cells (data not shown).

Discussion

In this study, we isolated a metastasizing human esophageal cancer cell line, T.Tn-AT1, from a non-metastasizing cell line, T.Tn. T.Tn-AT1 cells were highly tumorigenic and metastatic in nude mice when inoculated not only in s.c. tissue, but also at the orthotopic site (esophagus). T.Tn-AT1 cells were isolated *in vitro* on the basis of high adhesive ability to type 1 collagen and high cell-cell adhesion. Adhesive ability of cancer cells may have a dual role during the process of metastasis.[17] At the initial phase of carcinogenesis, loss of intercellular adhesion of epithelial cells promotes cell invasion into the stroma.[18, 19] However, in the metastatic process, multi-cellular aggregates of cancer cells may have a survival advantage under severe conditions, such as malnutrition and hypoxia.[19] Furthermore, multicellular aggregates of cancer cells are more resistant to the attack of inflammatory cells than single cells in the stroma, lymphatics, and blood vessels. Therefore, multi-cellular aggregates of cancer cells may be more efficient in forming metastasis than single cells.[18] Tomlinson *et al*. recently reported that overexpression and over-function of E-cadherin in inflammatory breast carcinoma cells enhanced the tumorigenicity and pulmonary lymphovascular emboli of the cells.[17] *In vitro* growth potential of T.Tn-AT1 was similar to that of the parental T.Tn. Thus, *in vitro* growth potential of the cancer cells was not directly associated with the metastatic potential of the tumor in nude mice. This observation is consistent with our previous findings.[15]

In this experiment, we also isolated another subclone, T.Tn-Mgr1, with high migrating ability *in vitro*. However, T.Tn-Mgr1 cells formed small tumors, which showed weak invasiveness and did not show metastatic potential in nude mice s.c.

Table 4. Genes that are more than 3-fold differentially expressed in T.Tn and T.Tn-AT1 cells

Gene name	Definition	Expression level		Expression ratio	
		T.Tn	T.Tn-AT1	T.Tn/ T.Tn-AT1	T.Tn-AT1/ T.Tn
AB033010	*Homo sapiens mRNA for KIAA1184 protein*	14.074	0.2829	49.748321	0.02010118
AF055376	*Homo sapiens short form transcription factor C-MAF (c-maf)*	0.5983	0.2021	2.9604156	0.33779041
AF070529	*Homo sapiens clone 24525*	0.7542	0.1742	4.3295063	0.23097322
AK001803	*Homo sapiens cDNA FLJ10941 fis, clone OVARC1001243*	0.589	2.1376	0.2755427	3.62920204
AK022962	*Homo sapiens cDNA FLJ12900 fis, clone NT2RP2004321*	0.8965	0.2306	3.8876843	0.25722253
AK023040	*Homo sapiens cDNA FLJ12978 fis, clone NT2RP2006321*	0.5048	0.1426	3.539972	0.28248811
AL080151	*Homo sapiens mRNA; cDNA DKFZp434D024*	0.4691	0.1169	4.0128315	0.2492006
AL117587	*Homo sapiens mRNA; cDNA DKFZp434M098*	1.7617	0.5714	3.0831292	0.3243458
AL137698	*Homo sapiens mRNA; cDNA DKFZp434C1915*	1.4501	0.4501	3.2217285	0.31039239
AL137708	*Homo sapiens mRNA; cDNA DKFZp434K0322*	3.6002	0.871	4.1334099	0.241931
NM_000216	*Homo sapiens Kallmann syndrome 1 sequence (KAL1)*	2.2818	0.3406	6.6993541	0.14926812
NM_000669	*Homo sapiens alcohol dehydrogenase 1C (class I), γ polypeptide (ADH1C)*	13.709	4.0443	3.3896348	0.29501703
NM_000860	*Homo sapiens hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD)*	15.122	5.0479	2.995721	0.33380946
NM_000867	*Homo sapiens 5-hydroxytryptamine (serotonin) receptor 2B (HTR2B)*	1.0423	0.293	3.5573379	0.28110909
NM_001266	*Homo sapiens carboxylesterase 1 (monocyte/macrophage serine esterase 1) (CES1)*	5.1352	1.3873	3.7015786	0.27015501
NM_001676	*Homo sapiens ATPase, H+/K+ transporting, nongastric, α polypeptide (ATP12A)*	2.2047	0.5252	4.1978294	0.23821835
NM_001974	*Homo sapiens egf-like module containing, mucin-like, hormone receptor-like sequence 1 (EMR1)*	0.5087	0.1416	3.5925141	0.2783566
NM_002487	*Homo sapiens necdin homolog (mouse) (NDN)*	6.8189	0.6857	9.9444363	0.10055874
NM_002899	*Homo sapiens retinol binding protein 1, cellular (RBP1)*	4.6166	1.3374	3.4519216	0.28969371
NM_002909	*Homo sapiens regenerating islet-derived 1 α (pancreatic stone protein, pancreatic thread protein) (REG1A)*	2.4951	0.2794	8.9302076	0.11197948
NM_003467	*Homo sapiens chemokine (C-X-C motif), receptor 4 (fusin) (CXCR4)*	1.393	0.3802	3.6638611	0.27293611
NM_003708	*Homo sapiens microsomal NAD+-dependent retinol dehydrogenase 4 (RODH-4)*	0.3169	1.2303	0.2575795	3.88229725
NM_004598	*Homo sapiens sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) (SPOCK)*	4.079	0.8321	4.902055	0.20399608
NM_004665	*Homo sapiens vanin 2 (VNN2), transcript variant 1*	0.3609	1.5182	0.2377157	4.20670546
NM_005070	*Homo sapiens solute carrier family 4, anion exchanger, member 3 (SLC4A3)*	0.5052	0.1491	3.38833	0.29513064
NM_005218	*Homo sapiens defensin, β 1 (DEFB1)*	1.9858	0.6324	3.1401012	0.31846107
NM_006729	*Homo sapiens diaphanous homolog 2 (Drosophila) (DIAPH2), transcript variant 156*	0.9109	0.165	5.5206061	0.18113953
NM_006891	*Homo sapiens crystallin, γ D (CRYGD)*	0.2413	1.5305	0.1576609	6.3427269
NM_012331	*Homo sapiens methionine sulfoxide reductase A (MSRA)*	0.3879	1.2607	0.3076862	3.25006445
NM_016002	*Homo sapiens CGI-49 protein (LOC51097)*	3.5063	0.8298	4.225476	0.23665973
NM_019000	*Homo sapiens hypothetical protein FLJ20152 (FLJ20152)*	2.6379	0.6804	3.8769841	0.25793245
NM_030806	*Homo sapiens chromosome 1 open reading frame 21 (C1orf21)*	4.2218	1.0662	3.9596699	0.25254631
NM_032495	*Homo sapiens lung cancer-associated Y protein (LAGY), transcript variant 1*	1.0367	0.204	5.0818628	0.19677824
NM_032955	*Homo sapiens allograft inflammatory factor 1 (AIF1), transcript variant 1*	10.058	0.1781	56.47333	0.01770747

These observations suggest that one prominent property of cancer cells *in vitro*, such as invasiveness, is not sufficient to produce metastasis *in vivo*. Fidler previously proposed that successful metastatic cells should be a "decathlon champion."[20] Thus, our observations are consistent with his hypothesis.

We examined the activity or the expression of major molecules that were reported to be associated with invasion and metastasis, such as MMPs, TIMPs, E-cadherin, and β-catenin, in T.Tn cells and the subclones, T.Tn-Mgr1 and T.Tn-AT1. However, there was no clear relation between the expression of these molecules and the *in vitro* or *in vivo* behavior of the cancer cells. Therefore, we compared the expression profiles of 9206 genes in non-metastasizing T.Tn cells and metastasizing T.Tn-AT1 cells by cDNA microarray analysis. As we expected, the gene expression profiles of T.Tn cells and T.Tn-AT1 cells were very similar. Only 34 among 9206 genes were picked up as genes that were more than 3-fold differentially expressed in T.Tn and T.Tn-AT1 cells.

Gene expression analysis by the microarray technique may have several benefits for cancer research. The first is the ability to determine the total gene expression profile and to compare the expression patterns among samples without focusing on individual genes. For such an experiment, a microarray containing all the transcripts of all human genes (approximately 100,000–200,000 transcripts, including alternative splicing variants) might be necessary. The second benefit of the microarray analysis is the abilty to screen or to pick up differentially expressed genes of interest, as an alternative to conventional methods, such as subtraction hybridization and differential display. Although we conducted cDNA microarray

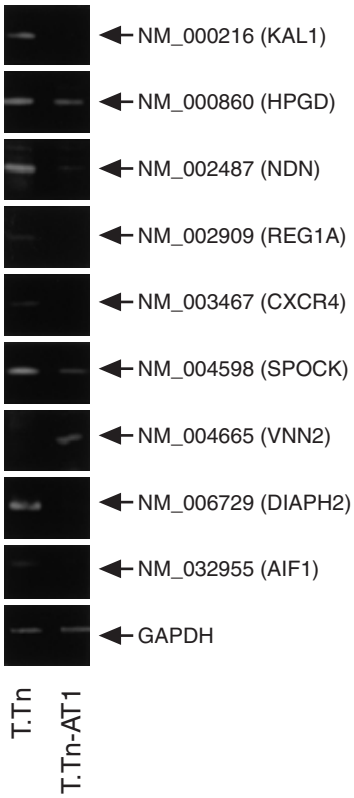

Fig. 6. Expression levels of the candidate genes in T.Tn and T.Tn-AT1 determined by semi-quantitative RT-PCR. *GAPDH* was used as an internal control.

analysis only once in two cell clones in this experiment, we utilized the microarray technique to screen for differentially expressed genes in the metastasizing human esophageal cancer cell line, T.Tn-AT1.

We then re-examined the expression levels of 14 of the 34 differentially expressed genes in T.Tn and T.Tn-AT1 cells by RT-PCR. The 14 genes were selected by carefully examining the original expression levels and altered expression levels in microarray analysis, and by reviewing the literature concerning these genes. We confirmed that 8 of the 14 genes were down-regulated, and one gene was up-regulated in T.Tn-AT1 cells. However, the expressions of the other five were not altered. Although the expression level of *AB033010* gene according to the microarray was approximately 50-fold greater in the parental T.Tn cells than that in T.Tn-AT1 cells, there was no difference in the expression of *AB033010* gene in the two cell lines by semi-quantitative RT-PCR. These discrepancies might be due to technical error or a technical limitation of the microarray analysis employed in this experiment. Therefore, a second or third screening to select genes for further examination, as we did with RT-PCR, may be generally necessary.

Among the 9 differentially expressed genes, 5 genes (*KAL1*,[21] *CXCR4*,[22] *SPOCK*,[23] *VNN2*[24] and *DIAPH2*[25]) encode proteins that are associated with adhesion and migration of inflammatory cells and cancer cells. Furthermore, *HPGD*[26] and *AIF1*[27] are also associated with inflammation. *HPGD* encodes 15-hydroxyprostaglandin dehydrogenase, which is reported to reduce the biological activity of prostaglandin.[28] *AIF1* has been found as an up-regulated gene of macrophages and neutrophils in cardiac allografts.[26] *REG1A*[27] and *NDN*[29] may regulate cell growth and differentiation. Thus, all 9 genes could be associated directly or indirectly with metastasis formation of esophageal SCC.

We are currently examining the expression levels of these putative metastasis-related genes in human esophageal cancer tissues (T1a and T1b) with or without lymph node metastasis. Moreover, in order to obtain direct evidence for the implication of the candidate genes in the metastatic potential of esophageal cancer cells, we are transfecting T.Tn or T.Tn-AT1 cells with sense- or antisense-oriented cDNA of the candidate genes, and are examining the alteration of their metastatic potential.

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