Transcriptome profiling of invasion phenotype associated with epithelial-mesenchymal transition in esophageal cancer

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Abstract

The esophageal cancer (ESC) is one of the ten-leading cancers in Taiwan with highly metastatic potential. The aim of this study was to identify relevant alterations of gene expression associated with the invasive phenotype of ESC. To reduce heterogeneity and to obtain data on genes specifically involved in invasive mechanisms, we have established a highly invasive ESC subline (CE48t/VGH) through 10 passages of in vitro Matrigel selections. This subline exhibited increases of invasion and migration abilities with the ascending generations, in accompany of morphological alteration from epithelial type to mesenchymal type, indicating an epithelial- mesenchymal transition (EMT) occurred during this Matrigel selection. In support of this finding, five molecular EMT markers were also examined accordingly. Affymetrix cDNA microarray was used to compare the transcriptomes between the parental and the EMT-associated invasion subline. Heretical cluster and algorithmic analyses of the microarray dataset revealed that several functional pathways associated with these phenotypic alterations. Among these 10 genes were examined for the potential differential expressions between parental and subline by RT-PCR or western blot analysis. In which, TKT, MYH9, Filaming A, Fibronectin, Gp96, Slug and IAGAP1 were found over-expressed in the invasion subline, suggesting a positive association in the regulation of EMT-associated invasion. In conclusion, results of this study provide molecular knowledge in the EMT-associated metastasis in ESC.
Background

Epidemiology of esophageal cancer (ESC)

Worldwide, esophageal cancer (ESC) is one of the ten-leading cancers of incidence (the 8th) and morality (the 6th) [1]. There were 15560 new cases and 13940 death cases in United states 2007 [2]. ESC is prevalent in China, with ~250,000 new cases diagnosed yearly, [3], and contributes to about half of the world's cases [4]. In Taiwan (2007), the incidence of ESC was the 9th leading cancer. ESC was mainly found in male. It is 6th leading cancer in male, and the death rate is increasing in the recent year [5, 6]. Esophageal cancer is one of the least studied cancers worldwide. At before, the research of esophageal was finding the important changes in the epidemiologic patterns associated with this disease, but now sciences often keep an eye on the diagnosis, staging, and treatment of this neoplastic condition have led to small but significant improvements in survival [7]. So do us, we want to find the esophageal cancer associated genes for the treatment in our research.

The esophagus is an organ in vertebrates which consists of a muscular tube through which food passes from the pharynx to the stomach. It is usually 25-30 cm long which connects the mouth to the stomach. ESC are cancers arising from the esophagus. There are more than 90 percent of esophageal cancers which are either squamous cell carcinomas or adenocarcinomas [8], but the other carcinomas, melanomas, leiomyosarcomas, carcinoids, and lymphomas may develop in the esophagus as well in several special cases. In Taiwan, almost 90% ESC patients were squamous cell carcinomas [6].

Most patients with esophageal cancer (74 percent) have dysphagia, and 17 percent report odynophagia at the time of diagnosis [8]. Weight loss is also common (occurring in 57 percent of patients) and is an independent indicator of a poor
prognosis if there is a loss of more than 10 percent of body mass [9]. Although longstanding gastroesophageal reflux disease is not uncommon in this group of patients (diagnosed in 21 percent), 14 percent of Americans have identical symptoms [10], and the vast majority of these people will never have esophageal cancer. Dyspnea, cough, hoarseness, and pain (retrosternal, back, or right obtained and typically shows a stricture or ulceration of the esophagus. The tumor surface may be fragile and bleed, causing hematemesis. Compression of local structures occurs in advanced disease, leading to such problems as superior vena cava syndrome.

The mean age of ESC patients was 67.3 years, and the risk increase with age [8, 11]. The cervical esophagus is an uncommon site of disease. There are a number of risk factors for ESC [7]. Some subtypes of cancer are linked to particular risk factors. The risk factors contain age, gender, heredity, tobacco, Human papillomavirus (HPV), particular dietary substances, other cancer’s metastasis…et cetera [7, 12, 13]. Substantial alcohol intake, especially in combination with smoking, greatly increases the risk of squamous cell carcinoma (but not adenocarcinoma) [13, 14], and may account for more than 90% of all cases of squamous cell carcinoma of the esophagus in the developed world [14]. The combination of smoking and alcohol abuse is associated with a similarly increased risk of head and neck cancer (HNC) [15].

Poor prognosis and high invasion / metastasis of ESC

ESC is classified according to the 2002 American Joint Committee on Cancer tumor–node–metastasis classification system, which takes into account the characteristics of the primary tumor, regional nodal metastases, and distant metastases. Overall, more than 50 percent of patients have unresectable or metastatic disease at the time of presentation. Among patients who are undergoing primary surgery, 13 to 20 percent have stage I disease, 14 to 27 percent stage IIA disease, 7 to 16 percent
stage IIB disease, and 40 to 54 percent stage III disease [11, 16, 17]. The stage at presentation appears to be relatively similar for adenocarcinomas and squamous-cell carcinomas [11]. ESC may spread rapidly; 14 to 21 percent of submucosal cancers (T1 lesions) and 38 to 60 percent of cancers that invade muscle (T2 lesions) are associated with spread to lymph nodes [11, 16]. At the clinical diagnosis, more than 50 percent of ESC patients have either unresectable tumors or radiographically visible metastasis [15]. After complete surgical removal of the tumor, the five-year survival rate exceeds 95 percent for stage 0 disease, and is 50 to 80 percent for stage I disease, 30 to 40 percent for stage IIA disease, 10 to 30 percent for stage IIB disease, and 10 to 15 percent for stage III disease [18-20]. Patients with metastatic (stage IV) disease who are treated with palliative chemotherapy have a median survival of less than one year [21]. Survival rates for esophageal cancer are poor; 75% of patients die within 1 year after diagnosis, and the 5-year survival rate is only 5–10% [22].

Because of esophagus' site on anatomical, most ESC are diagnosed at an advanced stage, and curing the disease is thus highly unlikely, especially by surgery alone [23]. The advanced stages are characterized by lymph node metastases and invasion to adjacent organs. Distant lymph node metastasis appears relatively early in patients with ESC because of anatomical characteristics that more lymphatic vessels run longitudinally than horizontally in the esophageal submucosal layer. Direct invasion of esophageal tumor cells into adjacent organs, especially the trachea or aorta, makes a complete resection impossible [24]. More than 50% of patients with ESC have incurable disease at presentation because of metastases, locally advanced disease or their poor medical condition [25]. The poor prognosis of ESC is due in large part to the early-stage invasion and metastasis to adjacent tissue and distant organs [26]. The metastasis ability of ESC not only brings the high death rate, but also add
the difficulty to cure. Finding the biomarker of metastasis may increase the sensitive of metastatic ESC’s diagnosis and the effective of treatment. The prognosis of ESC must be increased.

Metastasis is consists of a long series of multiple steps. Metastatic cells will spread from the primary neoplasm to distant organs [27]. The process include detachment and migration away from the primary tumor tissues, local invasion of the surrounding matrix, intravasation, survival in the circulation, extravasation, survival, and proliferation at the metastatic site [28]. The previously reports showed many pathways that involve those steps. The pathways include mitogen-activated protein kinase pathway, epidermal growth factor receptor signaling, transforming growth factor-β (TGF-β) signaling, EMT…etc [29-34]. Especially that there are many evidences showed the relationship between EMT and esophageal cancer [35-40]. Those studies indicated that EMT may play an important role in esophageal cancer.

The Epithelial mesenchymal transition (EMT) is a process that EMT cells differ in various functional characteristics. EMT was originally described during embryonic developmental process [41], such as gastrulation, renal organogenesis, and the formation of neural crest [42]. Accumulating evidence showed that EMT is reactivated in any many diseases and those include cancer progression and metastasis [43]. It’s well-documented that the carcinoma cells progression into metastatic competence will change their adhesive properties, activates proteolysis, and become motile, allowing them to achieve distant tumor metastasis [44]. The cells undergone EMT will lose their epithelial properties such as cell polarity, cell contact and gain the mesenchymal characteristics [45]. The mesenchymal cells will be contributed to various fibrotic conditions, and processed tumor EMT associated tumor invasion [46]. The EMT process often accompanied several markers’ expression level changing. The
markers have been identified that are characteristic of either epithelial or mesenchymal cells. The epithelial markers include E-cadherin, claudins, occludins, γ-catenin, cytokeratin-8, -9 and -18…etc, and the mesenchymal markers include fibrinectin, vimentin, vitronectin, N-cadherin, FSP1…etc [47, 48]. Recent studies revealed that some transcription factors, including Snail, Slug, ZEB1, and SIP1, are involved in the stimulation of EMT [49-51]. The transcription factors also modulated by the classic signaling pathways including the WNT, TGF-β, Hedgehog, Notch, and receptor tyrosine kinase and the pathways are often disregulated in cancer and have been shown to induct EMT [47]. It’s indicated that the EMT conversion not only plays an important role in cancer metastasis but also in cancer growth.

**Genes related to ESC invasion / metastasis have not been well defined**

Besides EMT, there are many reports which told about the ESC metastasis, and they found a lot of metastasis related genes in ESC (Table 1) [52-59]. For example, Li-yan Xue1 confirm the ESC metastasis related genes Extracellular matrix protein (laminin-5γ2) and matricellular protein (SPARC) by tissue microarray [55]. Summary Stomatin-like protein 2 (SLP-2) is a novel cancer-related gene whose product promotes cell growth, tumorigenicity, and adhesion in human esophageal squamous. Overexpression can be regarded as a significant prognostic factor, with higher expression being found in lymph node metastasis [57]. E-cadherin plays a key role in cell-cell adhesion, and loss of E-cadherin is a hallmark of tumor progression fostering cancer cell invasion and metastasis. It may play a role in ESC [58]. TM4SF3 promotes esophageal carcinoma metastasis via upregulating ADAM12m expression [56]. All of the single gene reports lacked the all-round view in ESC metastasis research, so some authors study by microarray. There were three comparing methods
in the previous microarray reports. First, Yasuto Uchikado applied oligo-microarray analysis of 17086 genes to identify the genes related to lymph node metastasis in ESC. The samples of cancer and non-cancerous paired tissue were taken from 16 patients with ESC who underwent esophagectomy with lymph node dissection [52]. Second, these previous reports compared the high and low level of lymph node metastasis ESC by cDNA microarray and selected several metastasis related genes [53, 54]. Third, the metastasizing ESC cell line from a parental non-metastasizing cell line and in vitro selection by nude mouse orthotopic inoculation model were compared the expression profiles of 9206 genes by cDNA microarray analysis. The 34 genes showed more than 3-fold differential expression [59]. There is a defect in these reports. How could they explain that the genes’ expression difference were because of the differential in each samples or cell line changing in the xenograft metastasis process? If we compare the same cell line with the higher and lower metastasis ability than each other in microarray, this defect will be overcome.

**Potential invasive associated genes in ESC**

Since the common area of carcinogenic risk exposure, patients with ESC often subject to develop head neck cancer. In support of this concept, it has been reported that 11.4% ESC patients also had head neck cancer [60]. Therefore, it is possible that ESC and head neck cancer may share common invasive mechanism. Our laboratory has previously found several potential invasive associated genes in head neck cancer, including MYH9, TKT, Filamin A and GP96. It is worthwhile to examine where these genes also play a role in ESC invasion.

- **Myosin, heavy chain 9, non-muscle (MYH9)**

  Toothaker et al. (1991) [61] observed that antisera raised against the peptide made from the predicted amino acid sequence specifically reacted with a 224-kD
polypeptide in leukocyte cell lines, and the protein was upregulated during the induction of monocytic and granulocytic differentiation in these cells. The cellular myosin heavy chain may be the major contractile protein responsible for movement in myeloid cell lines because no mRNA for sarcomeric myosin heavy chains is detected in these cells.

MYH9 will bind to actin. They play a role in cell shape, motility and cell division. MYH9 polymorphisms are thought to contribute to glomerulosclerosis[62] and non-diabetic end stage renal disease in African Americans.[63]

Integrins are a family of αβ heterodimeric membrane receptors that mediate cell – cell, cell – extracellular matrix, and cell – pathogen interactions. The integrin LFA-1 (or αιβ2) is expressed on most leukocytes and plays a major role in regulating leukocyte adhesion and recruitment to damaged or infected tissues during inflammation. MYH9 mediates integrin LFA-1 de-adhesion during T lymphocyte migration.[64]

- Transketolase (TKT)

Transketolase is a thiamine-dependent enzyme that links the pentose phosphate pathway with the glycolytic pathway. The pentose phosphate pathway, which is active in most tissues, provides sugar phosphates for intermediary biosynthesis, especially nucleotide metabolism, and generates the biosynthetic reducing power for the cell in the form of NADPH. Transketolase is directly involved in the branch of the pathway that channels excess sugar phosphates to glycolysis, enabling the production of NADPH to be maintained under different metabolic conditions. NADPH is critical for maintaining cerebral glutathione, and thus it is likely that transketolase plays an important role in brain metabolism.
Filamin A (FLNA)

Actin-binding protein, or Filamin, is a 280-kD protein that crosslinks actin filaments into orthogonal networks in cortical cytoplasm and participates in the anchoring of membrane proteins for the actin cytoskeleton. Remodeling of the cytoskeleton is central to the modulation of cell shape and migration. Filamin A, encoded by the FLNA gene, is a widely expressed protein that regulates reorganization of the actin cytoskeleton by interacting with integrins, transmembrane receptor complexes, and second messengers.

In the present study, Dana Ravida identify Filamin A as a prominent phosphoprotein, previously observed in caveolin-1-expressing stably transfected MCF-7 human breast cancer (MCF-7/Cav1) cells[65]. Furthermore, caveolin-1 expression upregulates Filamin A mRNA and protein levels and that Filamin A is a caveolin-1-dependent target of Akt in IGF-I-stimulated cells. Caveolin-1 is highly expressed in many cancer cell lines, as demonstrated initially in human multidrug resistant cancer cells [65, 66] and in mouse metastatic prostate cancer cells,[67] Filamin A is a novel caveolin-1-dependent target in IGF-I-stimulated cancer cell migration.[68]

Heat-shock protein, 90-kd, beta,1 (Gp96/Grp94)

HSP90 proteins are highly conserved molecular chaperones that have key roles in signal transduction, protein folding, protein degradation, and morphologic evolution. HSP90 proteins normally associate with other cochaperones and play important roles in folding newly synthesized proteins or stabilizing and refolding denatured proteins after stress. HSP90B1 is an endoplasmic reticulum HSP90 protein. Other HSP90 proteins are found in cytosol and mitochondria.[69]
As described previously, Gp96 is a member of Hsp90 family that is localized in the lumen of ER. This protein was found plays a role in tumor-specific immunity in mouse. Tumor rejection antigen-1 shows homology to hsp90, endoplasmic reticular protein (ERP99), and glucose-regulated protein (Grp94). And multiple functions have been reported including chaperoning peptides to MHC class I molecules of dendritic cells and other antigen-presenting cells.
Study aim and approaches

On this study, we want to identify the invasive associated genes in ESC. Two approaches were designed to achieve the goal: (1) Global survey approach. To establish highly invasion sublines of the ESC cells and determined the differential transcriptome. (2) Candidate gene examination. To identify the potential invasive genes in ESC.
Results

Establishment of highly invasive subline of ESC cells

A human esopharyngeal squamous carcinoma cell line CE48T/VGH was used. The invasive sublines of the cells was established by selection from parental cells, which were cultured on the polycarbonate membrane coated with Matrigel in a Transwell invasion chamber. After incubating for 24 hours at 37°C, cells which migrated through the Matrigel were harvested and continuously cultured till confluence, as defined ten generation of the subline cells. Figure 1 shows the morphology of the cells. The appearance of pseudopods in highly invasive cells were found, which may function to be favorable in migration.

A human esopharyngeal squamous carcinoma cell line CE81T/VGH was also used. But the CE81 subline establishment process was not smooth as CE48, the invasion subline could not be establishment up to now. So we tried the other way to establish the invasion subline, we did the subcutaneous injection of the CE81 to nude mice. We derived the tumor tissue isolated of nude mice post-injection 9 days (Figure 2a), and did the primary culture to establish the new cell subline- nude mice inject 81 (NMI81). The morphology is showed in figure 2b. The NMI81 may be more malignant than parental CE81 and the feather may let the invasion subline establishment easier than before.

Verification of highly invasive phenotype in CE48 subline cells

In order to verify the invasive phenotype, Matrigel invasion assay was used. As shown in the Figure 3a, the parental, subline IV and subline XI’s invasion ability was be compared. The numbers of cells invaded to the lower chamber is higher in invasive subline. The invasive ability increasing fold change had shown in Figure 3b. This result suggests that the invasion ability were increased in subline and the invasion
ability was also elevated with the ascending generation.

Figure 4 determined the cell migration ability by migration transwell assay between parental, subline III, V and X. We can observe that the numbers of migration cells to transwell in the highly invasive subline were more than parental cells.

The Figure 5 also determined the cell migration ability by wound healing assay. We can observe that the six generation invasion subline’s wounded area was almost completely covered at 23 hr, whereas the CE48 parental cells were still moving toward into the area. The parental cells’ wounded was covered at 48 hr. It suggests that the migration ability were increased in subline. By those data, we can sure that the invasive subline is successfully established.

The Figure 6 shows the cell growth ability between parental, subline III and subline VI. It shows that the growth ability was decrease in subline, and the difference was remarkable increased in ascending days.

The colonogenic assay determined the colony formation ability between parental, subline III, V and X in Figure 7a. The Figure 7b has shown the quantitation data. The highly invasive cells have shown the lower colony formation ability than poor invasive cells. The colony formation and growth ability must be decreased in invasive subline establishment.

**EMT may play a role in subline establishment**

Morphology changing could be found in subline establishment. Figure 1 has shown the cell morphology of parental, subline III, V and X. Cells changed from a polygonal or round appearance to an elongated shape. The cell might lose the cell-cell adhesion ability and polarity. The morphology changed from epithelial cell type to mesenchymal type with subline generation increasing. Further more, we explore the several EMT markers and EMT associated transcription factors’ expression level
between parental, subline III, V and X by western blotting assay in Figure 8. The mesenchymal markers (Fibronectin and N-cadherin) and slug were increased in highly invasive subline. The epithelial marker (E-cadherin) expression was losses in sublines. The data suggest that the EMT must be undergone on subline establishment.

**Transcriptome profiling of the invasive genes by cDNA microarray**

The eight generation of ESC subline along with the parental cells were subjected to Affymetrix microarray analysis. A threshold of 2.5-fold up-regulation or down-regulation of gene expression was used. Sixty-six genes were differentially expressed (41 p-regulated and 25 down-regulated) (Table 3.) The functional classification of differentially expressed genes is listed in Table 4. Among the up-regulated genes, a substantial number were involved in cell adhesion/ metastasis/ angiogenesis / cytoskeleton (41.46%) and growth / cell cycle (15.15%). Among the down-regulated genes, a substantial number were involved in cell adhesion/ metastasis/ angiogenesis / cytoskeleton (28%) and immune response (28%).
Materials and Methods

Cell lines and cell culture

ESC cell lines, CE81T/VGH, CE48T/VGH and the nude mice injection 81 and esophagus normal cell CGESNK1 and CGESNK2 were used. These cancer cell lines were cultured in DMEM medium (Gibco BRL, CA, USA) with 10% FCS and 1% antibiotics. The two normal cells were obtained from Chang Gung hospital and cultured in KSFM medium. The nude mice injection 81 cells were derived from primary tumor samples isolated of mice injected subcutaneously with CE81T/VGH. Cells were incubated at 37°C in humidified 5% CO2-95% air.

Establishment of highly invasive subline and invasion assay

The subline of ESC cultured cells was established by several selection and culture from parental cells passing through a polycarbonate membrane coated with Biocoat Matrigel (Becton Dickinson Biosciences, Bedford MA) in a Transwell invasion chamber (NUNC, USA). Invasion assays were performed by using a Matrigel invasion chamber with a pore size of 8 μm (Becton Dickinson Biosciences, Bedford MA). The lower surface of the membrane was coated with 10μg/ml Matrigel for 2 h at 37°C, washed with PBS, and then blocked with 1% BSA for 1 hour at 37°C.

Transwell migration assay

Cell migration assays were performed by using Transwell polycarbonate chambers (Becton Dickinson Biosciences). After cell migration into lower chamber for 12 hours, cells were fixed with 1% glutaraldehyde (Sigma), stained with 0.5% crystal violet (Sigma). For quantitation of the migrated cells, cells were incubated for 30 minutes at 37°C, and eluted with 0.5 ml of Sorensen’s solution (9 mg of trisodium citrate in 305 ml of distilled water, 195 ml of 0.1 N HCl, and 500 ml of 95% ethanol). The absorbance was determined with a microtiter plate reader (Becton Dickinson
Labware) at 570 nm.

**Wound healing migration assay**

Cell migration were evaluated by *in vitro* wound healing assay. In brief, monolayer cultures of the cancer cells grown in fibronectin-coated dishes were “wounded” by a micropopett tip and then incubated in the presence of 1% FBS culture medium for 7 hours in a tissue culture incubator. Cell migration toward the wounded area were observed and photographed.

**RNA extraction**

Total RNA was extracted with TRIzol reagent (Gibco BRL) following the manufacturer’s instructions. The concentration, purity, and amount of total RNA were determined by ultraviolet spectrometry.

**Reverse transcription reaction Polymerase chain reaction (RT-PCR)**

The gene expression of potential invasive genes was analyzed by using RT-PCR. A mixture contained 50 mM Tris , 75 mM KCl, 3 mM MgCl₂, 400 ng of RNA, 0.2 ug oligo-dT, 10 mM DTT, 0.5 mM dNTP, 10 units of RNase inhibitor (RNaseOut, Invitrogen, CA, USA), and 50 units of reverse transcriptase (M-MLV, Invitrogen) were incubated in 37°C for 1 hour in a final 30 µl of reaction for cDNA synthesis.

To amplify specific genes, PCR reactions were carried out with 30 cycles of denaturation at 95°C for 40 sec, annealing at 56~60°C for 40 sec, and extension at 72°C for 1 min. The PCR products were analyzed by 1~2 % agarose gel electrophoresis, then stained with ethidium bromide, visualized and photographed by illuminating with 254 nm UV.

**Western blotting**

Cells were washed with PBS buffer and treated with trypsin. Cellular proteins were extracted by incubation for 30 min at 4°C with iced-cold CHAPS lysis buffer (10
mM Tris, pH 7.4, 1 mM MgCl₂, 1 mM EGTA, 150 mM NaCl, 0.5% CHAPS and 10% glycerol). Samples were centrifuged at 14,000 rpm for 30 min and the supernatant was harvested for protein quantification and western blotting analysis.

For western blot analysis, 30 µg of cellular protein was prepared. All samples were boiled at 95°C for 5 min, and subjected to 10% SDS-polyacrylamide gel for electrophoresis. The proteins on the electrophoretic gel were transferred to a nitrocellulose membrane and blocked with 5% non-fat milk in PBST solution (phosphate buffer saline plus 0.1 % Triton X-100). After being washed with PBST 10 mins twice, the membrane was incubated with 1:1000 dilution of first antibodies at room temperature for 2 hours. The membrane was washed again and incubated with secondary IgG antibody conjugated with horseradish peroxidase. Membrane was incubated with ECL developing solution (Amersham Pharmacia Biotech) and exposed to X-ray film. Actin expression was used as an internal control.

**Colony formation assay**

Transfected cells were seeded at the cell number of 1000~3000 per well of a 6-well plate and cultured routinely in DMEM medium (Gibco BRL) supplemented with 10% FCS and 1% antibiotics. After incubation of the cells in 37°C, 5% CO₂ incubator for 7~12 days, cell were stained with crystal violet (Sigma) for 15 minutes. After being washed with ddH₂O, the numbers of colony dormation were quantitaed.

**Growth assay**

The moderate cells seeded at 6-well plate and cultured routinely in complete mediums, and cultured for 24, 48, and 72 hr at 37°C in a humidified incubator containing 5% CO₂. Cell viability was determined by staining with 0.25% trypan blue.
Microarray hybridization and data analysis

Probes were generated using the procedures described by Affymetrix (Santa Clara, CA, USA) by the Cang Gung Memorial Hospital Microarray Core Facility and hybridized to an Affymetrix U133A Genechip (Affymetrix, CA, USA). The microarrays were evaluated as described by Affymetrix using a GeneArray 2500 confocal scanner (Affymetrix, CA, USA). The average signals from two sequential scans were calculated for each microarray feature. Background subtraction were carried out using the algorithms provided by Affymetrix Microarray Suite 5.0 (Affymetrix, CA, USA). Total gene expression signals of each array were scaled to 150 signal units to allow comparison between arrays.

Scaled data were imported into the TIGR Multiexperiment Viewer version 2.2 from the Institute of Genomic Research (Rockville, MD, USA). SAM were used to identify genes differentially expressed between N+ and N- samples (Tusher et al., 2001). Genes whose mean expression levels for both groups fell below background, estimated by the average of Bacillus subtilis gene signals, were discarded. The remaining signature set of genes were analysed using principal-components analysis (scaled for equal variance, results extracted by JScatter, a lab-based program, for visualization) and SVM. Crossvalidation of the original samples using a leave-one-out approach and a diagonal factor of 2 provided an estimate of the accuracy of the SVM algorithm, which were also used to predict the classes of four independent samples.
Table 1. There are many reports which told about the ESC metastasis, and they found a lot of metastasis related genes in ESC (table 1) [52-59].

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Function</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>With extro-cellular matrix affect factor</td>
<td>TM4SF3</td>
<td>Cytoskeleton</td>
<td>fascin</td>
</tr>
<tr>
<td></td>
<td>ADAM12m</td>
<td></td>
<td>CK14</td>
</tr>
<tr>
<td></td>
<td>TFPI-2</td>
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<td>CK4</td>
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<tr>
<td></td>
<td>SPARC</td>
<td>Metabolism</td>
<td>PLSCR1</td>
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<td></td>
<td>laminin-5γ2</td>
<td></td>
<td>DUSP1</td>
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<td>annexin I</td>
<td>Adhesion</td>
<td>TM7SF2</td>
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<td>FAS</td>
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<td>SLP-2</td>
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<td>Adhesion</td>
<td>E-cadherin</td>
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<td></td>
<td>caspase8</td>
<td>Immunity</td>
<td>Interleukin 1-β</td>
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<tr>
<td></td>
<td>VEGF</td>
<td></td>
<td>interleukin 6</td>
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Table 2. List of 66 genes differentially expressed in the highly invasive subline of oral cancer cell line as determined by Affymetrix microarray.

Up-regulated

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Fold change</th>
<th>Gene Title</th>
<th>Fold change</th>
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<tr>
<td>NNMT</td>
<td>3.282454</td>
<td>SERPINA1</td>
<td>2.744954</td>
<td>BLCAP</td>
<td>2.512605</td>
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<tr>
<td>IL8</td>
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<td>TOMM7</td>
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<td>H2AFB3</td>
<td>2.629062</td>
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<td>SGNE1</td>
<td>3.201254</td>
<td>LOC201725</td>
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<td>H2AFB1</td>
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</tbody>
</table>
### Down-regulated

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Fold change</th>
<th>Gene Title</th>
<th>Fold change</th>
<th>Gene Title</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAT1</td>
<td>0.399444</td>
<td>FEZ2</td>
<td>0.381291</td>
<td>ATP6V0A4</td>
<td>0.299816</td>
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<tr>
<td>HNRPA3</td>
<td>0.398016</td>
<td>PSG7</td>
<td>0.372311</td>
<td>GJA1</td>
<td>0.298772</td>
</tr>
<tr>
<td>NONO</td>
<td>0.394352</td>
<td>TSN</td>
<td>0.3721</td>
<td>PSG6</td>
<td>0.295537</td>
</tr>
<tr>
<td>PSG2</td>
<td>0.393792</td>
<td>CANX</td>
<td>0.362602</td>
<td>PSG3</td>
<td>0.288784</td>
</tr>
<tr>
<td>PTPLB</td>
<td>0.391071</td>
<td>UPK1B</td>
<td>0.360049</td>
<td>SCD</td>
<td>0.281335</td>
</tr>
<tr>
<td>SCD</td>
<td>0.388916</td>
<td>FKBP1A</td>
<td>0.332472</td>
<td>PSG9</td>
<td>0.273442</td>
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<tr>
<td>LAPTM4B</td>
<td>0.388912</td>
<td>FDFT1</td>
<td>0.327017</td>
<td>ADM</td>
<td>0.258024</td>
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<tr>
<td>PSG1</td>
<td>0.388335</td>
<td>TXNDC</td>
<td>0.317117</td>
<td>SCD</td>
<td>0.217693</td>
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<tr>
<td>ITGA6</td>
<td>0.387188</td>
<td>PSG9</td>
<td>0.30614</td>
<td>UPK1B</td>
<td>0.184275</td>
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<tr>
<td>MACF1</td>
<td>0.382854</td>
<td>VIL2</td>
<td>0.302985</td>
<td>PSG4</td>
<td>0.139016</td>
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</tbody>
</table>
**Table 3.** Representative subset and functional classification of 79 genes differentially expressed in the invasive oral cancer cell subline compared to parental cells with low invasive potential.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Total</th>
<th>Up-regulation</th>
<th>Down-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion/metastasis/angiogenesis/cytoskeleton</td>
<td>36.36% (24/66)</td>
<td>41.46% (17/41)</td>
<td>28% (7/25)</td>
</tr>
<tr>
<td>Immune response</td>
<td>15.15% (10/66)</td>
<td>7.31% (3/41)</td>
<td>28% (7/25)</td>
</tr>
<tr>
<td>Growth/ cell cycle</td>
<td>13.64% (9/66)</td>
<td>19.51% (8/41)</td>
<td>4% (1/25)</td>
</tr>
<tr>
<td>Transcription/ translation</td>
<td>12.12% (8/66)</td>
<td>7.31% (3/41)</td>
<td>20% (5/25)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>9.09% (6/66)</td>
<td>9.76% (4/41)</td>
<td>8% (2/25)</td>
</tr>
<tr>
<td>Metabolism</td>
<td>7.58% (5/66)</td>
<td>7.31% (3/41)</td>
<td>8% (2/25)</td>
</tr>
<tr>
<td>Development</td>
<td>3.03% (2/66)</td>
<td>4.88% (2/41)</td>
<td>0% (0/25)</td>
</tr>
<tr>
<td>Stress response</td>
<td>1.52% (1/66)</td>
<td>2.44% (1/41)</td>
<td>0% (0/25)</td>
</tr>
<tr>
<td>Others/ unknown</td>
<td>1.52% (1/66)</td>
<td>0% (0/41)</td>
<td>4% (1/25)</td>
</tr>
</tbody>
</table>
Table 5. Functional gene network associated with invasiveness of oral cancer cells.

<table>
<thead>
<tr>
<th>Function</th>
<th>Map</th>
<th>genes/total objects</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion</td>
<td>Cell adhesion Role of tetraspanins in the integrin-mediated cell adhesion</td>
<td>7/37</td>
<td>2.863e-04</td>
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<tr>
<td></td>
<td>Cell adhesion Chemokines and adhesion</td>
<td>11/93</td>
<td>4.317e-04</td>
</tr>
<tr>
<td></td>
<td>Cell adhesion Alpha-4 integrins in cell migration and adhesion</td>
<td>5/31</td>
<td>4.691e-03</td>
</tr>
<tr>
<td>Cell adhesion / Cytoskeleton</td>
<td>Cytoskeleton remodeling Regulation of actin cytoskeleton by Rho GTPases</td>
<td>4/23</td>
<td>8.551e-03</td>
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<tr>
<td></td>
<td>Cell adhesion ECM remodeling</td>
<td>6/51</td>
<td>9.504e-03</td>
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<tr>
<td></td>
<td>Cell adhesion Endothelial cell contacts by non-junctional mechanisms</td>
<td>4/24</td>
<td>9.979e-03</td>
</tr>
<tr>
<td></td>
<td>Cell adhesion Endothelial cell contacts by non-junctional mechanisms</td>
<td>4/24</td>
<td>9.979e-03</td>
</tr>
<tr>
<td>Immune response</td>
<td>Immune response Alternative complement pathway</td>
<td>7/34</td>
<td>1.637e-04</td>
</tr>
<tr>
<td></td>
<td>Immune response Lectin induced complement pathway</td>
<td>6/41</td>
<td>3.182e-03</td>
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<tr>
<td></td>
<td>Immune response Classical complement pathway</td>
<td>6/43</td>
<td>4.068e-03</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Cholesterol biosynthesis</td>
<td>7/21</td>
<td>5.229e-06</td>
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<tr>
<td>Cell cycle</td>
<td>Cell cycle Sister chromatid cohesion</td>
<td>5/21</td>
<td>7.359e-04</td>
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</table>
Figure 1. Establishment of highly invasive ESC cell sublines. ESC cell lines, CE48T/VGH were used. The sublines of cultured cells were established by selection by parental clines, which were cultured on polycarbonate membrane coated with Matrigel in a Transwell invasion chamber. After incubating for 24 hours at 37°C, cells which migrate through the Matrigel were harvested and continuously cultured till confluency. Now, the selection process was reported to establish six generation. We showed the morphology of parental and sublines.
Figure 3. Invasion ability of ESC invasive sublines. (A) Cell numbers were counted in lower chamber after seeding $10^5$ ESC cells on Matrigel for 24 hours. CE48T/VGH cells and the invasive subline. The invasive subline content of cell numbers. (B) The invasive ability increasing fold change was determined by subline invasive cell number divided by parental invasive cell number of invasion assay.
Figure 4. Migration ability of ESC invasive sublines. Transwell migration assay determine cell migration ability in ESC cells. Seeded $10^5$ ESC cells into upper chamber of transwell polycarbonate chambers well of a 24-well plate. After cell migration into lower chamber, cells were fixed and stained with 0.5% crystal violet (Sigma).
Figure 5. Migration ability of ESC invasive sublines. Cell migration were evaluated by *in vitro* wound healing assay. The ESC cell line CE48 and invasion subline VI were seeded 1.5 x 10⁶ cells on 6-well plate and the cells were be “wounded” by amicropopett tip and then incubated in the presence of 1% FBS culture medium for 7 hours in a tissue culture incubator. Cell migration toward the wounded area were observed and photographed.
Figure 6. Growth ability of ESC invasive sublines of CE48 cells. The parental, I3 and I6 cells were seeded $1 \times 10^5$ at 6-well plate and cultured routinely in complete mediums, and cultured for 24, 48, and 72 hr at 37°C in a humidified incubator containing 5% CO$_2$. Cell viability were determined by staining with 0.25% trypan blue.
**Figure 7.** The parental, I3, I5, I10 cells were seeded 1000, 2000, 3000 per well of a 6-well plate and cultured routinely in DMEM medium (Gibco BRL) supplemented with 10% FCS and 1% antibiotics. After incubation of the cells in 37°C, 5% CO₂ incubator for 7~12 days, cell were stained with crystal violet (Sigma) for 15 minutes. After being washed with ddH2O, the numbers of colony dormation were quantitaed. (B) Quantitation of cells was performed using Sorensen’s solution as described in the Materials and Methods section CE48T/VGH cells and the invasive subline.
Figure 9. CDNA microarray were identified the gene expression difference between the parental cells and highly invasive sublines. It was used to determine the invasion associated genes. Representative subset and functional classification of 66 genes differentially expressed in the invasive ESC cell subline compared to parental cells with low invasive potential.
**Figure 11.** The previous research in our lab. The proteomes of parental and highly invasive sublines displayed in 8~16% polyacrylamid gradient gel. Cellular protein were fractionated to cytosol, membrane and nuclear proteins and the differential proteomes in parental and highly invasive sublines were separated by 8~16% gradient gel stained with G250. Proteins with differential expressions in parental (P) and invasive sublines (I) were picked up for further identification by MALDI-TOF. The identified protein list in table 2. (From:長庚大學生技所邱清旗 碩士論文2005年)
**Figure 14.** The genes who appeared in both microarray and proteome screening selected as invasion candidate genes.
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