Molecular Characterization of Invasive Subpopulations from an Esophageal Squamous Cell Carcinoma Cell Line

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Abstract. *Background: Once diagnosed, esophageal cancer has a very low overall 5-year survival rate. This study investigates the mechanisms behind the invasiveness and severity of esophageal squamous cell carcinoma (ESCC). Materials and Methods: Transwell invasion chamber was used to subdivide one Taiwanese ESCC cell line, CE81T/VGH, into sublines (CE81T-0, CE81T-1, CE81T-2, CE81T-3, and CE81T-4) in four rounds of assays; the most invasive were identified, and various factors related to their invasiveness measured. Results: CE81T-1, CE81T-2, CE81T-3 and CE81T-4 sublines were significantly more invasive than the parental cells (CE81T/VGH) and CE81T-0 subline. CE81T-1 and CE81T-4, the sublines we chose to study further, had significantly greater colony-forming ability (3.5- to 2.7-fold) and wound migrating activity (1.95- to 2.6-fold) than the parental cells in* vitro (p<0.01). They also displayed greater tumorigenesis in immunodeficient BALB/c Foxlnn mice than the parental cells. We found an inverse correlation between expression of tissue inhibitor of metalloproteinase-2 and invasive ability, and a significant positive correlation between expressions of matrix metalloproteinase-1, vimentin, and p-Src (pY416) in these cell lines and their invasiveness (all p<0.05). Conclusion: The subline model may be used to study the molecular and genetic mechanisms underlying the invasion and metastasis of ESCC.*

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In 2002, esophageal cancer was the sixth most deadly cancer, with 386,000 deaths reported worldwide (1). In Taiwan, esophageal squamous cell carcinoma (ESCC) was the sixth most common cause of cancer deaths in males in 2007 (2, 3). The prognosis of ESCC is generally poor (<15% overall 5-year survival rate) due to extensive local invasion and frequent lymph node metastasis (4, 5).

Invasion and metastasis begin when tumor cells first attach to the surrounding extracellular matrix (ECM) and produce matrix-degrading enzymes before finally migrating through the degraded matrix to the blood stream (6). During this remodeling activity, degradation of the extracellular matrix (ECM) is thought to result from the combined action of several proteolytic enzyme systems and motility factors (7, 8). In order to study the mechanisms associated with different phases of

invasiveness, the transwell invasion chamber (9) has been successfully used to select and analyze the invasiveness of cell lines and sublines of cancer cell lines known to cause skin, lung, and ovary cancer (10-12). However, to our knowledge, this model has not been applied in studies of cell lines of esophageal cancer. This study used this model to characterize the invasive and migration ability of one Taiwanese ESCC cell line and its sublines through *in vitro* and *in vivo* studies.

Materials and Methods

Cell lines and culture conditions. The Food Industry Research and Development Institute (Hsinchu City, Taiwan; http://www.firdi.org.tw/index.htm) provided the esophageal cancer cell lines CE146T/VGH (BCRC 60167), CE81T/VGH (BCRC 60166), and CE48T/VGH (BCRC 60165), which were obtained from 50-, 57-, and 58-year-old Taiwanese males, respectively. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL) and kept at 37°C in a 5% CO_2/95% air atmosphere.

Selection of invasion cells by transwell invasion chamber. Subpopulations from the CE81T/VGH esophageal cancer cell line were selected by the membrane invasion culture system (MICS) or BD BioCoat™ Matrigel™ Invasion Chamber (MA, USA). Briefly, cells were suspended in DMEM containing 10% FBS and seeded into the wells. After incubation at 37°C for 72 h, the inserts were removed. The cells that had invaded the membranes and had become attached to the lower-chamber compartments were harvested and allowed to proliferate for a second round of selection. For MICS selection, the sublines of the first-round selection in the upper and lower well chambers were designated as CE81T1-0 and CE81T1-1, respectively, and sublines from the second, third, and fourth rounds of selection were designated as CE81T1-2, CE81T1-3, and CE81T1-4, respectively. The parental line in the first series was designated CE81T1. For BD BioCoat™ Matrigel™ Invasion Chamber selection, the sublines of the first-round selection in the upper and lower well chambers were designated CE81T2-0 and CE81T2-1, respectively, and sublines from the second, third and fourth rounds of selection were designated CE81T2-2, CE81T2-3, and CE81T2-4, respectively. The parental line in the second series was designated CE81T2.

In vitro invasion assay. The MICS was used to measure the invasion capacity of each cell line. Assays were performed using polycarbonate membranes (Falcon HTS Fluoro Blok™ insert; BD Biosciences, Franklin Lakes, NJ, USA). Invasion assay was performed using membranes with uniformly-coated reconstituted basement gel (Matrigel; BD Biosciences, Bedford, MA, USA). The insert was placed in a 24-well culture dish (Falcon) containing DMEM and 10% NuSerum (BD Biosciences). In each well, $5×10^4$ cells were resuspended in DMEM containing 10% NuSerum and then seeded into the upper wells of the chamber. After 36 h of incubation at 37°C, cells that had migrated or had invaded the membrane were stained with the fluorogenic compound 4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA). Invading cells were counted manually using three random microscopic fields per well. The DAPI fluorescence of nuclei was visualized by excitation at 330-385 nm with a 420 nm barrier filter. Images were captured using a Nikon inverted fluorescence microscope (Nikon ECLIPSE TE300; Tokyo, Japan) with attached camera at ×100 magnification and processed using ImagePro Plus Version 5.0 software (Media Cybernetics, MD, USA). Experiments were repeated in triplicate.

Genotypic analysis and molecular cytogenetic analysis. The genomic background of CE81T1 and its sublines (CE81T1-1 and CE81T1-4) were studied using a Genome-wide Human Array SNP6.0 (Affymetrix, CA, USA). The single nucleotide polymorphism (SNP) genotype reactions were performed using 500 ng of genomic DNA from each sample according to the manufacturer's instructions. Genotyping was performed by the National Genotyping Center at Academia Sinica, Taipei, Taiwan, ROC (http://ngc.sinica.edu.tw). After washing, hybridization intensities were captured by GeneChip Scanner 3000 (Affymetrix). Intensity data were analyzed using Genotyping Console Version 2.1 (Affymetrix) and Partek Genome Suite (Partek Incorporated, MS, USA).

Soft agar colony-forming assay. Cells exhibiting exponential growth were suspended in complete growth medium containing 0.33% Bacto-agar (Sigma A-6013 Type 1 Low EEO; Sigma, Detroit, MI, USA) and overlaid on 0.5% agarose gel in 30-mm dishes (10^4 cells/dish). The dishes were maintained at 37°C in a humidified incubator (5% CO_2, 95% O_2) for two weeks. During this period, the medium was changed every three days. The numbers of visible colonies (>50 μm) were counted under a microscope. All experiments were performed in quadruplicate.

Wound-healing assay. The cancer cells were cultured in 6-well plates and grown in medium containing 10% FBS until cell monolayers approached confluence then a scratch was carefully made using a plastic pipette tip to produce a linear 'wound' in the cell monolayer of each well. The cultures were photographed before and then 24 h after incubation at 37°C. The number of cells that had migrated into the cell-free zone was recorded. The experiments were performed in triplicate, and cells were counted in a double blind fashion by at least two researchers.

Zymography for gelatinase. All cell lines were cultured in DMEM with 10% FBS until they reached subconfluence. They were then washed with phosphate-buffered saline (PBS) and cultured in DMEM without serum for 24 h. The conditioned media were collected, concentrated with Amicon Ultra (Millipore, Carrigtwohill, Co. Cork, Ireland) by repeated centrifugation at 2000×g for 10 mins then mixed with Laemmli SDS sample buffer (without β-mercaptoethanol) containing 25 mM Tris/HCl (pH 6.8), 10% glycerin, 1% SDS, and 0.1% bromophenol blue for electrophoresis. Electrophoresis was performed on 10% polyacrylamide gels containing 0.1% SDS and gelatin at a final concentration of 0.1% (w/v). The gels were then washed three times in wash buffer containing 50 mM Tris/HCl (pH 7.6), 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 2.5% TritonX 100 for 10 mins to remove the SDS. During this process, proenzymes activated autocatalytically. The gels were then incubated for 24 h in a reaction buffer (50 mM Tris/HCl (pH 7.6), 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.1% NaN_3, and 1% TritonX 100) and stained with Coomassie blue. The gels were destained in a 45% (vol/vol) methanol/10% (vol/vol) acetic acid solution until transparent bands appeared on the blue background. The DMEM with 0.1% FBS was used as a positive control.

RNA extraction and RT-PCR. Zymographic findings were confirmed by RT-PCR. Total RNA was isolated from the CE81T/VGH and its sublines using TRIzol reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions then amplified using the Superscript First-Stand Synthesis System for reverse transcription-PCR (Promega, WI, USA). The primer sequences and sizes of the PCR products of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), matrix metalloproteinase-1 (*MMP-1*), and tissue inhibitor of mmp-2 (*TIMP-2*) genes were reported (13, 14). Each RT-PCR was performed in a thermal cycler (GeneAmp PCR System 9700; GeneAmp, MA, USA). The number of cycles was increased to 23 for *GAPDH* and 27 for *MMP-1* to ensure generation of submaximal products. The amplified products were separated by electrophoresis with 100 bp DNA Ladder (BTCM100; BERTEC, Taichung, Taiwan, ROC) in 1.5% agarose gels and then visualized under UV light after 0.5 μg/ml ethidium bromide staining.

Flow cytometry for vimentin. Cells (1×10^5) were fixed with 4% formaldehyde in PBS for 10 min at room temperature then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Cells were incubated for 30 min with mouse monoclonal antibody (VI-RE/1) to vimentin (Phycoerythrin) (ABcam, Cambridge, UK) and analyzed using a Becton Dickinson FACScan and CellQuest software. Nuclei were stained by incubating the cells with 6 μmol/l DAPI. The cells were observed under a Fluoview FV 500 confocal microscope (Olympus, Tokyo, Japan) and data processed by a Fluoview FV 500 analysis system (Olympus).

Western blot analysis for p-Src and TIMP-2. Western blot analysis was performed using polyclonal rabbit phospho-Src antibody (pY416; Cell Signaling Technology, Danvers, MA, USA) and polyclonal rabbit TIMP-2 antibody (SantaCruz, San Diego, CA, USA). Isolated cells were homogenized in lysis buffer (150 mmol/l NaCl, 0.1% sodium dodecyl sulfate, 10 mmol/l EDTA, and 1% NP40, 1× protease inhibitor; Roche, Mannheim, Germany) and 50 mmol/l Tris-HCl (pH 7.5). To analyze phospho-Src (pY416), NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) was added to the sample and sample reducing agent (Invitrogen). After centrifugation at 13,000 × rpm at 4°C for 15 mins, the supernatant was collected, and total protein was evaluated by BCA protein assay (Pierce, Rockford, IL, USA). After boiling for 5 mins with 5×SDS-PAGE loading buffer (25 mMTris (pH 6.8), 1% SDS, 10% glycerol, 17% β-mercaptoethanol, and 0.2% bromophenol blue), samples containing 40 μg of protein per lane were separated on 10% acrylamide gel or 4-12% NuPAGE (invitrogen, Carlsbad, USA) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the separated protein was transferred to a PVDF membrane (Pall, Pensacola, Florida, USA). After blocking in 5% milk-TBST solution, the membrane was sequentially incubated with primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG antibody 1:5000; Pierce) using a chemiluminescence detection system (ECL Western blotting detection reagents; Millipore, Billerica, USA). Finally, the membranes were washed and exposed to film. An internal control protein, GAPDH, was re-probed with an anti-GAPDH polyclonal antibody (Ab FRONTIER, Seoul, Korea) using the above procedures after 20 min treatment with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl) followed by repeated washing in distilled water.

Tumorigenicity in BALB/c Foxlnn mice. Eight-week-old male BALB/c Foxlnn mice were acquired from the National Laboratory Animal Center (Taipei, Taiwan, ROC). The mice were housed under strict pathogen-free conditions and given sterile food and water. The Committee on Animal Research at Kaohsiung Medical University approved all procedures (No. 96102). *In vitro* cultured CE81T1 and its sublines (CE81T1-1 and CE81T1-4) cells (1×10^6 cells/0.2 ml Hanks buffer (Gibco/BRL)) were injected subcutaneously into the dorsal region of each mouse. Each tumor cell line was injected into six different mice. Tumor development was assessed by weekly measurements of the tumor mass for five weeks.

Experimental metastasis. Two sublines (CE81T1-1 and CE81T1-4) with invasive characteristics different from those of the parental line of CE81T1 were washed and re-suspended in HBSS. The CL1-4 cell line of lung cancer, a gift from the laboratory of Dr. Yang Pan-Chyr at the National Taiwan University Hospital (12), was used as positive control. A single-cell suspension containing 10^6 cells in 0.2 ml Hanks buffer was then injected into the lateral tail veins of eight-week-old BALB/c Foxlnn mice. Each tumor cell line was injected into seven mice. After 8 weeks, the mice were asphyxiated with CO_2 then examined for metastasis to the lungs, liver, brain, and esophagus. The organs were removed and fixed in 10% formalin fixative. The number of esophageal tumor colonies was counted under a dissecting microscope. The representative tumors were removed, fixed, and embedded in paraffin then sectioned into 4-μm layers and stained with hematoxylin and eosin (H&E) for histological analysis.

Statistical analysis. All *in vitro* and *in vivo* experiments were conducted in triplicate or in quadruplicate. Data are presented as means±S.E. of three or more independent experiments. All data were analyzed by two-tailed unpaired Student *t*-test, and $p < 0.05$ was considered statistically significant.

Results

Selection and characterization assay in ESCC
Comparison of invasiveness between three Taiwan ESCC cell lines. After the three separate experiments, the mean (±standard error (SE)) numbers of invading cells in the CE48T/VGH and CE81T/VGH cell lines were 44±2 and 44±2, respectively, which were significantly higher than that in the CE146T/VGH (2±0.1) (both $p < 0.001$) (Figure 1A). Thus, the CE81T/VGH cell line was used to select the sublines by MICS due to its highly active invasiveness. The mean numbers of CE81T1-1, CE81T1-2, CE81T1-3, and CE81T1-4 cells that had invaded through the transwell invasion chamber were 254±47, 138±38, 198±51, and 101±17, respectively, which were significantly higher than the mean numbers of CE81T1 (43±7) and CE81T1-0 (17±4) cells (all $p < 0.05$) (Figure 1B).

Growth of CE81T1 and sublines in soft agar. The colony-forming rate was significantly higher in the highly invasive sublines than in the parental cells (CE81T1-1 353±9% and CE81T1-4 265±9% *vs*. CE81T1 100±8%, respectively; both $p < 0.01$) (Figure 2).
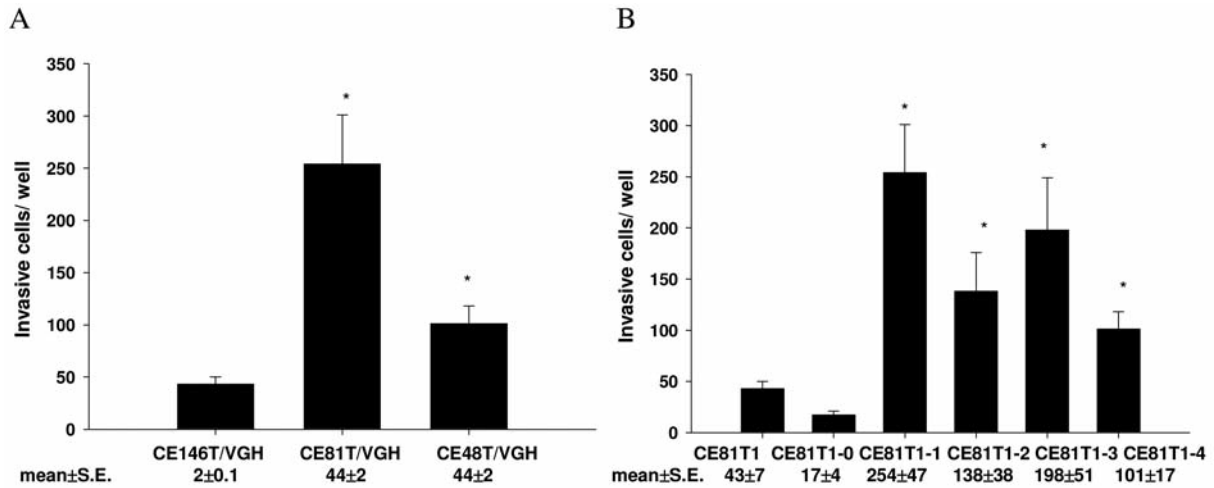
Figure 1. *Selection and invasion characterization assay in CE81T/VGH and its sublines (CE81T1-0, CE81T1-1, CE81T1-2, CE81T1-3, and CE81T1-4). A: Invasion assay in three Taiwanese esophageal squamous cell carcinoma (ESCC) cell lines *p<0.001 compared to CE146T/VGH. B: In vitro invasion activity of CE81T1 and its sublines *p<0.05 compared to CE81T1 and CE81T1-0. Experiments were repeated in triplicate.*
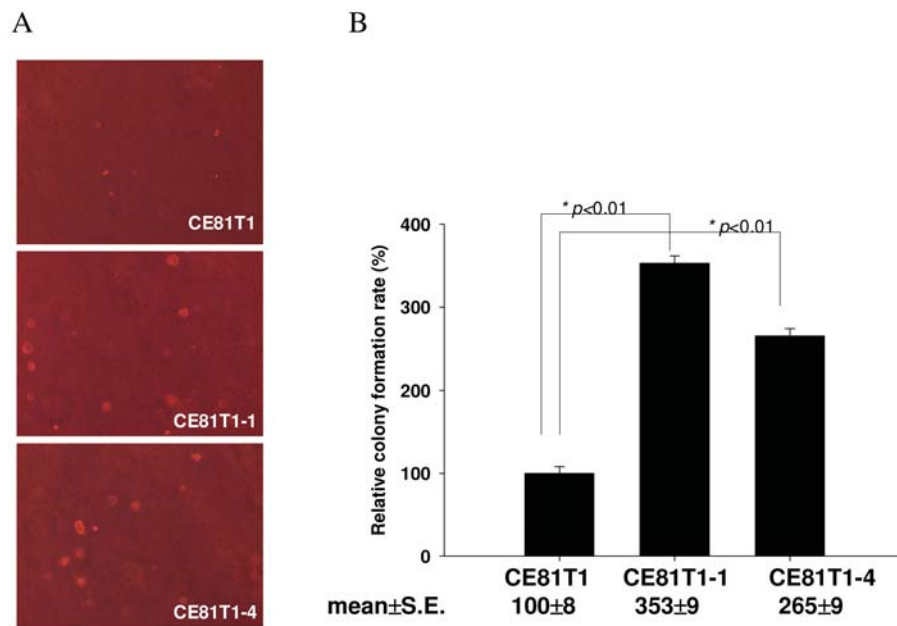

Figure 2. *Soft agar colony formation of the CE 81T/VGH cell line and its sublines. A: Photomicrographs of colonies were taken three weeks after plating (original magnification ×100). B: Quantitative analyses of colony formation (quadruplicate).*

Identifying genomic aberrations specific to the CE81T1 cell line and its sublines. Genome-wide SNP genotyping with Affymetrix SNP6.0 array was performed to assess loss of heterozygosity (LOH) and DNA copy number changes for CE81T1, CE81T1-1, and CE81T1-4. The rate of genotype concordance between CE81T1-1 and CE81T1 was 99.35%, and that between CE81T1-4 and CE81T1 was 99.15%, indicating that CE81T1-1 and CE81T1-4 originated from the same CE81T1 cell line (Table I).

Characterization of CE81T1 and its sublines at the molecular level

Gelatine zymographic study of MMP activities of proteins secreted in the CE81T1 and its sublines. Although similar MMP-2 activities (~72 kDa) were noted in CE81T1, CE81T1-1, and CE81T1-4 (Figure 3A), the MMP activities of CE81T1-1 and CE81T1-4 were obviously present at approximately 41 kDa (MMP-1) in contrast to parental cells (CE81T1) (Figure 3A).
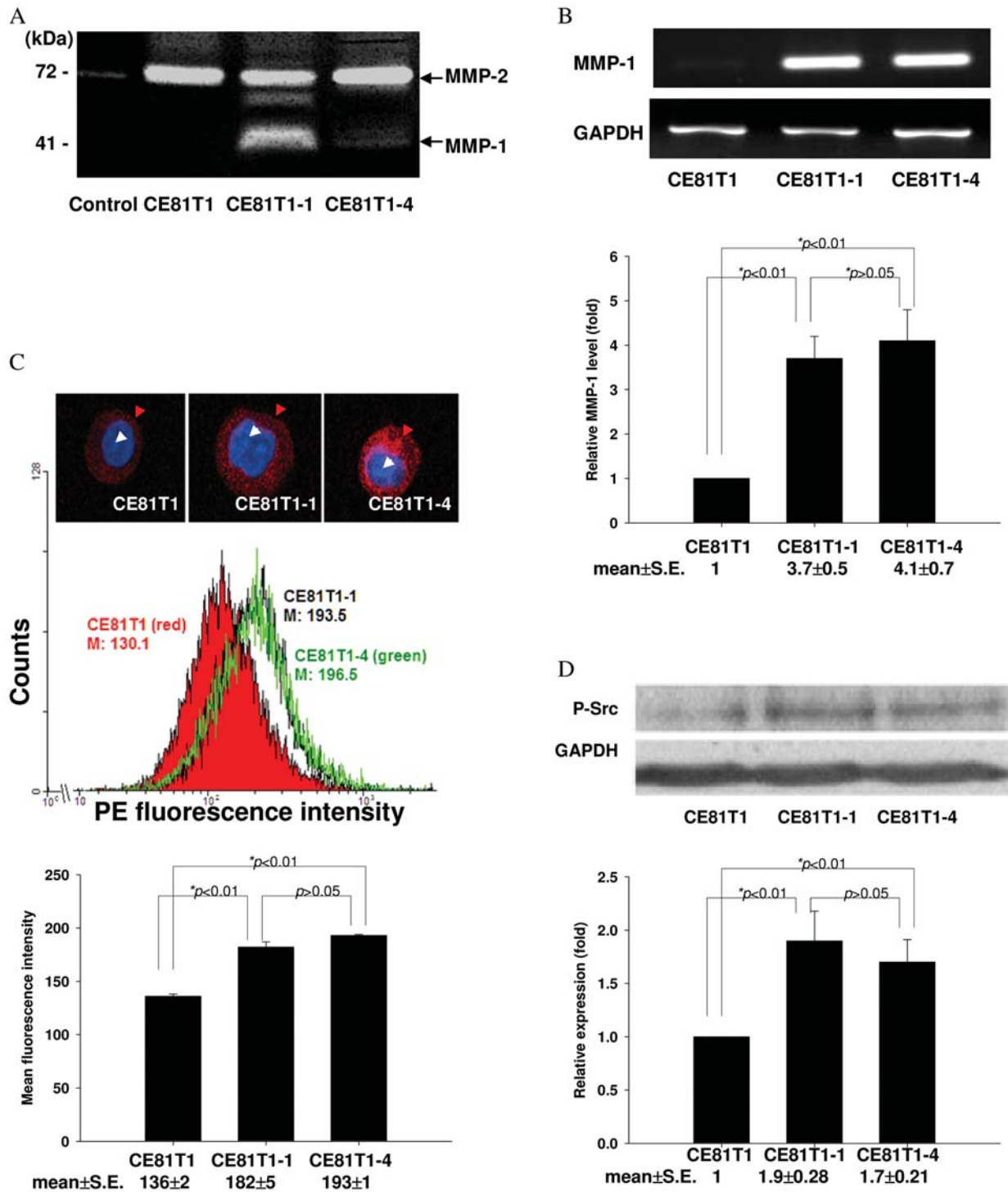
Figure 3. *Molecular characteristic features of CE81T/VGH and its sublines. A: Functional expression of MMP-1 in CE81T1 and its sublines (CE81T1-1 and CE81T1-4). Zymographic analysis of secreted gelatinase activity (five separate experiments). Media were collected from three sublines and from 0.1% culture medium (Lane 1) as a positive control. B: Expression of MMP-1 in CE81T1 and its sublines by RT-PCR analysis (triplicate). C: Comparison of vimentin expression of CE81T1 and its sublines stained by PE (phycoerythrin). Photomicrographs of cells were taken (original magnification ×1500). White arrow indicates cell nucleus with blue (4,6-diamidino-2-phenylindole, DAPI) fluorescence stain; red arrow indicates vimentin stained with red (phycoerythrin, PE) fluorescence stain; M, mean red fluorescence intensity of fixed cells stained with PE from respective cultures in one representative experiment. Data are presented as means±S.E. of triplicate assays. D: Expression of phosphorylated-Src (p-Src) in CE81T and its sublines by Western blot analyses (three separate experiments).*
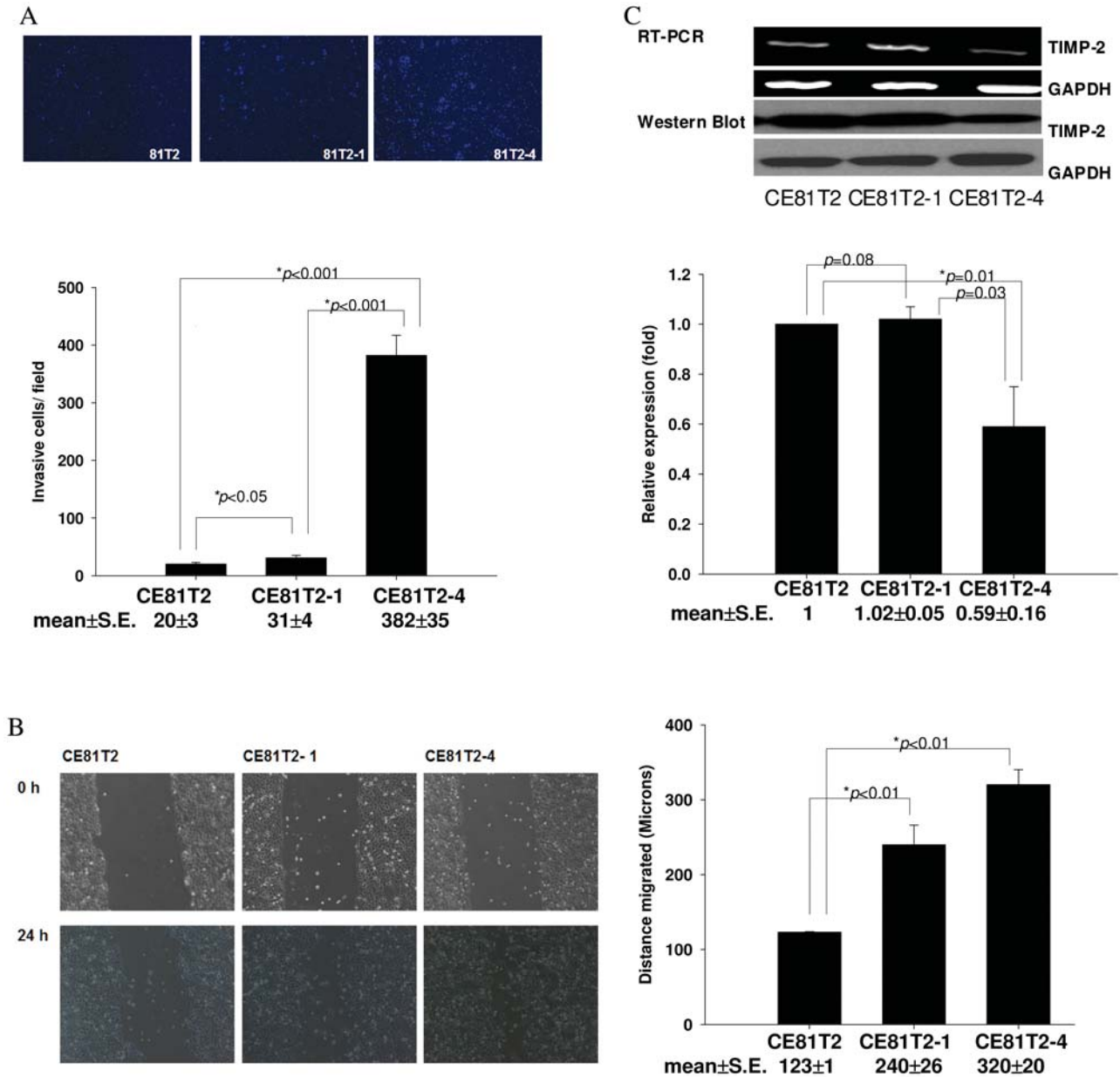
Figure 4. *Selection and characterization assays in CE81T/VGH and its sublines (second selection). A: In vitro invasion activity of CE81T2 and its sublines (CE81T2-1 and CE81T2-4) (original magnification, ×100). B: Confluent migration of CE81T2 and its sublines by wound-healing assay (original magnification, ×100). C: RT-PCR and Western blot analyses of TIMP-2 in CE81T2 and its sublines (three separate experiments).*

MMP-1 expression at the mRNA level in the CE81T1 cell line and its sublines. The gelatin zymography results were further confirmed by a RT-PCR analysis of CE81T1 and its sublines (CE81T1-1 and CE81T1-4). *MMP-1* mRNA was expressed in CE81T1-1 and CE81T1-4 but only weakly expressed in CE81T1. Comparisons with CE81T1 revealed that, after correcting relative to GAPDH, the CE81T1-1 and CE81T1-4 sublines exhibited 3.7±0.5-fold and 4.1±0.7-fold increases in expression of *MMP-1*, respectively (Figure 3B).

Flow cytometric comparison of vimentin expression in CE81T1 and its sublines. Vitmentin expression was higher in CE81T1-1 and CE81T1-4 than in the parental cells (CE81T1). Elite-ESP flow cytometry (Miami, FL, USA) and E4win-ESP analysis Software programs were used for vimentin analysis in 10,000 cells. The mean vimentin-PE fluorescence of CE81T1-1 and CE81T1-4 were significantly higher than that in the parental cells (182±5 and 193±1 *vs.* 136±2, respectively; all $p<0.01$) (Figure 3C).
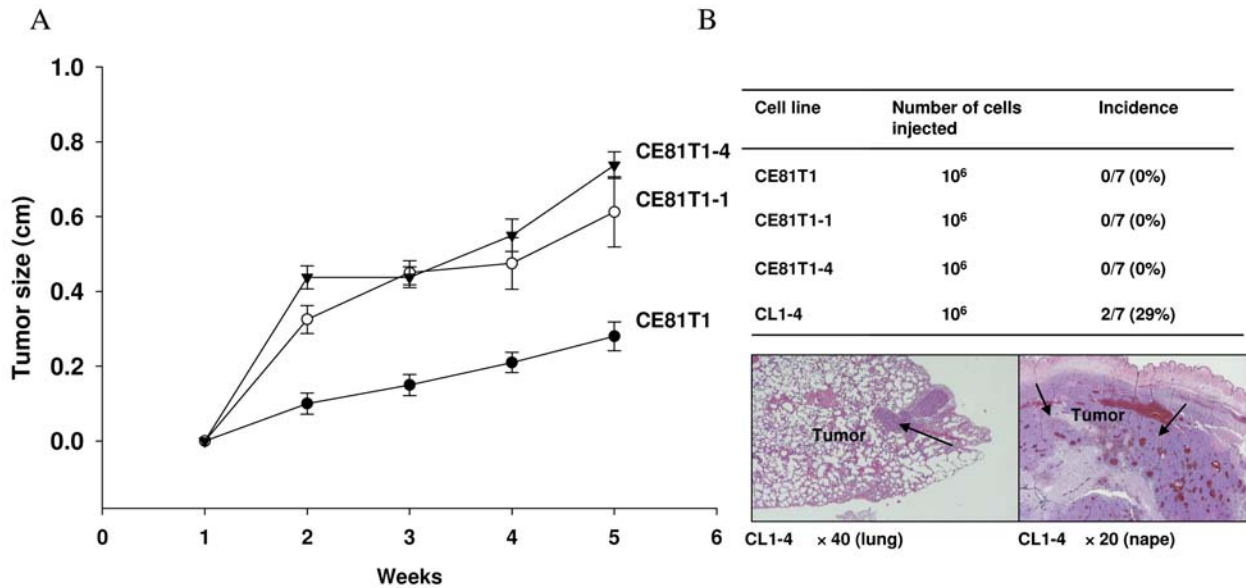
Figure 5. *In vivo characterization study. A: Tumorigenicity of esophageal cancer cells in BALB/c Foxlnn mice. B: Incidence of experimental metastasis of CE81T1 cell line and its sublines (CE81T1-1 and CE81T1-4) in BALB/c Foxlnn mice. CL1-4: human lung adenocarcinoma cell line was used as a positive control (12). Histological analysis of CE81T1 cell line and its sublines in BALB/c Foxlnn mice. The lungs and tumor were removed, fixed in formalin, and embedded in paraffin. H&E staining was then performed on 4-μm-thick sections of lung and tumor tissue.*

Western blot analysis of phosphorylated-Src (p-Src) expression in CE81T1 and its sublines. The p-Src (pY416) activity was examined by Western blot analysis using a monoclonal antibody recognizing tyrosine phosphorylation of Y416. After correcting relative to GAPDH, the p-Src (pY416) activity of CE81T1-1 and CE81T1-4 was 1.9 ± 0.28-fold and 1.7 ± 0.21-fold respectively, higher than that in the parental cells (CE81T1) (Figure 3D).

Re-selection of CE81T and its sublines using separate transwell invasion chamber and their characterization. After the passage of ~30 generations, an apparent generation-to-generation decrease in invasive ability was observed in CE81T1 and its sublines. Therefore, the human CE81T cell line and its sublines were reselected using procedures described previously. The second parental line was referred to as CE81T2. The CE81T2-1 and CE81T2-4 cell lines were significantly more invasive than their parental line CE81T2 (31 ± 4 and 382 ± 35 *vs.* 20 ± 3, respectively; both $p<0.001$) (Figure 4A).

Wound-healing test analysis of CE81T2 and its subline motility. The distances between cells were measured 24 h after they were separated by scratching in the wound-healing test. The distances were then subtracted from their original position after separation to calculate their migration distance. As of 24 h into the healing process, the CE81T2-1 and CE81T2-4 cells had migrated further than the CE81T2 cells (in microns, 240 ± 26 and 320 ± 20 *vs.* 123 ± 1, respectively) (all $p<0.001$) (Figure 4B).

RT-PCR and Western blot analysis of TIMP-2 expression in CE81T2 and its sublines. *TIMP-2* mRNA was strongly expressed in CE81T2 and CE81T2-1 but weakly expressed in CE81T2-4. Moreover, TIMP-2 protein expression was significantly lower in CE81T2-4 cells than in CE81T2 and CE81T2-1 cells (both $p<0.05$) (Figure 4C).

Tumor growth and metastasis in nude mice. One dose of CE81T1, CE81T1-1, and CE81T1-4 cells was injected into the subcutaneous tissue in the dorsal regions of six T-cell-deficient BALB/c Foxlnn mice in each group. From the second to the fifth week, the tumors in mice injected with CE81T1-1 or CE81T1-4 cells grew more rapidly than those injected with CE81T1 cells (Figure 5A). Metastasis was also assessed by injecting CE81T1 cells and these of its two sublines into the tail veins of T-cell-deficient mice. Eight weeks after injection, the tumors in mice revealed no metastasis to the lung or other organs. Positive control mice received injections of CL1-4 cells into the tail vein. Histological study revealed metastasized tumors (one in lung and the other in the nape) in two (29%) mice (Figure 5B).

Discussion

To date, the molecular mechanisms underlying esophageal cancer metastasis remain poorly understood. In this study, highly invasive cell sublines of CE81T/VGH, an ESCC cell line, were selected for future research. This study used MICS

Table I. *Confirming the identities of ESCC sublines with high-density SNP array. To confirm the identities of CE81T1, CE81T1-1, and CE81T1-4, genome-wide SNP genotyping (A) and concordance analysis (B) were performed as described in the Materials and Methods. Overall, the data quality is good for each sample, as determined by contrast QC and QC call rate (A). Concordance analysis was performed for SNPs with calls in paired sample and reference. The results indicated that CE81T1, CE81T1-1, and CE81T1-4 were almost identical (B).*

A.

Sample	Called gender	Call rate		Contrast QC				QC Call rate			
				Random	Nsp	Sty	Overlap		Nsp	Nsp/Sty overlap	Sty
CE81T1	Male	98.23652	2.36	2.36	2.63	2.51	2.46	96.19	96.02	93.88	97.17
CE81T1-1	Male	97.631	2.34	2.34	3.21	2.68	2.71	93.98	93.44	90.98	95.38
CE81T1-4	Male	98.14835	2.29	2.29	2.73	2.49	2.79	94.97	93.44	93.56	96.24

Item	Definition
Contrast QC	Computed contrast QC for all QC SNPs (SNP 6.0 only).
Contrast QC (Random)	Contrast QC for 10 k random autosomal SNPs (SNP 6.0 only)
Contrast QC (Nsp)	Contrast QC for QC 20 k SNPs on Nsp fragments (SNP 6.0 only).
Contrast QC (Sty)	Contrast QC for QC 20 k SNPs on Sty fragments (SNP 6.0 only).
Contrast QC (Nsp/Sty Overlap)	Contrast QC for QC 20 k SNPs on both an Nsp and Sty fragment (SNP 6.0 only).
QC Call rate	Computed QC call rate for all QC SNPs
QC Call rate (NSP)	(SNP 5.0, SNP 6.0 only)
QC Call rate (Nsp/Sty Overlap)	(SNP 5.0, SNP 6.0 only)
QC Call rate (Sty)	(SNP 5.0, SNP 6.0 only)

B.

Sample	Reference	# SNPs Called	# Concordant SNPs	% Concordance
CE81T1-1	CE81T1	873977	868306	99.35
CE81T1-1	CE81T1-4	873965	864000	98.86
CE81T1-4	CE81T1	878119	870641	99.15

SNPs Called: number of SNPs that were called in paired sample and reference and included for concordance analysis.
Concordant SNPs: number of SNPs whose genotype calls were identical in paired sample and reference.

with transwell invasion chamber for selecting sublines. The MICS, which was developed in 1984 (9), and extracellular matrices such as Matrigel ™, enable researchers to accelerate the screening for cell invasion that is fundamental to tumor cell metastasis. The system has been used to analyze cancer cells of the ovary, skin, and lungs (10-12).

The invasive characteristics of examined cancer cell subpopulations were confirmed by *in vitro* studies such as *in vitro* invasion assays, soft agar colony-formation assay, and wound-healing test, which were then verified by *in vivo* studies using a BALB/c Foxlnn (immunodeficiency) mouse model to evaluate tumor growth rate. Genomic analysis using a genome-wide Affymetrix SNP6.0 array indicated that the parental cell line and two sublines were virtually identical, which suggests that CE81T1-1 and CE81T1-4 were derived from CE81T1 and that the difference in invasive ability of the sublines was probably due to epigenetic changes rather than genetic mutation. These findings have not been examined in previous studies of other organ cell lines (10-12).

The reason for selecting two CE81T/VGH subpopulations (CE81T1 and CE81T2) for a series of subsequent experiments in this study was that, after the passage of ~30 generations, the invasive characteristics of CE81T1-1 and CE81T1-4 had largely diminished by the time the *in vitro* invasion experiments were repeated. These experimental results were consistent with those observed in an earlier study of the human lung adenocarcinoma cell line by Chu *et al.* (12) (personal communication). This phenomenon was satisfactorily explained by examining the genome-wide SNP data to determine what changes in the sublines, in comparison with the parental cells, were epigenetic rather than genomic. The invasion profiles of subpopulations in the first (CE81T1-1 and CE81T1-4 *vs.* CE81T1) and second (CE81T2-1 and CE81T2-4 *vs.* CE81T2) rounds of selection also differed (Figure 1B and 4A). The sublines of the first series had irregular round-to-round differences in invasive ability, and the sublines of the second series displayed increasing round-to-round invasive ability. Because the wells of the 24-well

invasion chamber used in the first series were relatively small, the selection process for the four rounds was time-consuming (~3 months). Therefore, a 6-well invasion chamber was subsequently used to accelerate the selection process and to collect a larger number of cells. In this series, four rounds of selection required ~3 weeks. Therefore, the different invasion profiles of those two selection procedures have two possible explanations. Firstly, the time required for environmental factors to affect the invasive ability of cells may have been longer in the first series than in the second. Secondly, since the cell line is heterozygous, the average invasiveness can be decreased by slow proliferation of cells, even though they may actually be more invasive. Since the invasive abilities of the sublines exceeded that of their parental cells in both rounds, functional differences were compared only in parental cells and their sublines in this study.

The *in vivo* study revealed that the tumor growth rate in BALB/c Foxlnn mice subcutaneously treated with CE81T1-1 or CE81T1-4 cells was more rapid than that in mice treated with CE81T1, but distant metastasis was not noted in any of three groups of mice that received tail-vein injections. However, in the positive control experiment of the highly metastatic CL1-4 (12) lung cancer cell line, two out of seven mice revealed lung and nape metastases, indicating that the lack of metastasis in mice treated with CE81T was not due to methodological differences. The reason for the lack of metastasis is unclear; however, the data are consistent with clinical reports of local recurrence in 44% (32/72) of esophageal cancer patients (15) but in fewer than 10% of lung cancer patients (16).

The MMPs are a family of proteinases known to degrade many ECM components and to play a central role in tumor invasion (17). Analysis and characterization of MMP activity using the gelatine zymographic assay revealed no difference in the functional expression of ~72 kDa (MMP-2) between the parental cell line and two sublines. However, the significant functional difference of ~41 kDa gelatinase activity in those cell lines suggested the presence of MMP-1 (18). To confirm the gelatin zymography results, CE81T1 and its sublines (CE81T1-1 and CE81T1-4) were analyzed by RT-PCR and *MMP-1* mRNA was expressed in CE81T1-1 and CE81T1-4 but only weakly expressed in CE81T1. We also measured MMP-1 activity by gelatin zymography in CE81T2 and its sublines and found MMP-1 protein levels were higher in CE81T2-1 and CE81T2-4 than CE81T2 (data not shown), which was similar to the observation in CE81T1 and its sublines. Of the collagenases, MMP-1 is one of the most capable of cleaving native fibrillar collagen (19). A possible cause of the aggressive tumors in subpopulations with high MMP-1 activity is the ability of these enzymes to degrade many components of the basement membrane and connective tissue and thus possibly play a critical role in the invasive and metastatic processes of ESCC.

The most invasive sublines (CE81T1-1 and CE81T1-4) exhibited significantly higher expressions of both vimentin and p-Src (pY416) than their parental CE81T1 cells did. Vimentin is a type III intermediate filament protein often expressed in particularly invasive epithelial carcinomas associated with poor prognosis (20). In many types of cancer, including colon, breast, pancreatic, and lung cancer, Src activity occurs during the invasion and metastasis processes (21). Activation of Src family kinases in human cancer may occur through various mechanisms and is frequently a critical event in tumor progression (22), but its role in ESCC has not been fully elucidated.

Conclusion

Transwell invasion chambers were successfully used to select particularly invasive sublines of the Taiwanese ESCC cell line CE81T/VGH. The invasive characteristics of these cancer cell subpopulations were confirmed both by *in vitro* and *in vivo* assays. Future studies by the Authors may apply functional arrays to those sublines in order to further clarify the molecular and genetic mechanisms underlying the invasion and metastasis of this disease.

Acknowledgements

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