Tumorinitiating cell population (TIC), also known as cancer stem cells, are regarded widely as a specific subpopulation of cells needed for cancer initiation and progression. TICs have yet to be identified in esophageal tumors that have an increasing incidence in developed countries. Here, we report a CD90+ cell population found in esophageal squamous cell carcinoma (ESCC), which is endowed with stem cell–like properties and high tumorigenic and metastatic potential. mRNA profiling of these cells suggested pathways through which they drive tumor growth and metastasis, with deregulation of an Ets-1/MMP signaling pathway and epithelial–mesenchymal transition figuring prominently. These cells possessed higher self-renewal activity and were sufficient for tumor growth, differentiation, metastasis, and chemotherapeutic resistance. CD90+ TICs were isolated and characterized from ESCC clinical specimens as well as ESCC cell lines. In freshly resected clinical specimens, they represented a rare cell population, the levels of which correlated with strong family histories and lymph node metastasis. Our results prompt further study of this CD90+ population of esophageal TICs as potential therapeutic targets. Cancer Res; 73(7); 2322–32. ©2013 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the major histologic subtype of esophageal cancer. The disease is one of the most common malignancies and ranks as the sixth leading cause of cancer-related deaths worldwide, with a dismal 5-year survival rate of only 20% to 30% after curative surgery (1, 2). ESCC is characterized by its remarkable geographic distribution; with distinctly high incidences and mortality rates particularly in China and other countries in Asia (3). Despite advances in diagnosis and treatment of ESCC, the disease remains devastating due to late diagnosis, aggressive nature of the cancer (metastasis), and a limited understanding of the cellular and molecular mechanisms underlying initiation and progression of ESCCs.

Cancer is hierarchically organized and composed of heterogeneous population of cells, among which numerous studies have now provided evidence of the existence and importance of a tumor-initiating cell (TIC) or cancer stem cell (CSC) compartment (4). This fraction of tumor cells shares many similarities with normal stem cells, such as self-renewing and differentiation capacity. In addition, TICs are also highly tumorigenic and are able to survive adverse microenvironments. With both stem cell–like and cancer properties, these TICs are believed to be the small fraction of cells responsible for initiation and maintenance of the entire tumor mass. From a clinical point of view, the main concern with TICs is their resistance to conventional treatments, a feature that has now been extensively shown to be an underlying cause of tumor recurrence. This would require that we rethink the way we diagnose and treat tumors, as our objective would have to be now focused specifically on TICs that fuels tumor growth. Thus, there is a need to identify and characterize the properties of these TICs to develop more efficient therapies against it. Thus far, studies have found TIC populations in ESCCs to be marked by a p75 neurotrophin receptor (p75NTR) or CD271 or CD44 surface phenotype (5–7). These cells were found to display classic TIC features including the ability to initiate tumor, self-renew, and resist standard chemotherapy. However, studies thus far have only been limited in looking at ESCC cell lines but not freshly resected ESCC clinical samples. Furthermore, little is known about the underlying molecular mechanisms of TICs in ESCCs and the role of TICs in ESCC metastasis. We hypothesized that there is a


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subset of TICs capable of forming metastasis in addition to tumorigenesis.

In this study, through integrative RNA-Seq analysis on 3 matched pairs of patient-derived ESCC clinical specimens and their adjacent nontumor (NT) tissue counterparts, we identified CD90 to be moderately and commonly overexpressed in ESCCs. CD90 (also known as Thy-1) is a surface glycoprotein of 25 to 28 kDa, which is expressed on the cytoplasmic membrane of different cell types. CD90 expression in freshly resected primary ESCC clinical samples ranged from 2.4% to 10%, whereas expression in normal or premalignant dysplastic clinical samples was significantly less (0%–1.4%). CD90+ esophageal TICs not only displayed enhanced ability to initiate tumor, self-renew, differentiate and resistant chemotherapy but also conferred an enhanced metastatic potential. Furthermore, CD90 expression was also found to have prognostic value in patients with ESCCs, as higher CD90 expression was significantly associated with a strong family history of ESCCs and higher incidences of lymph node metastasis. Subsequently, we explored possible deregulated pathways by which this specific population of cells mediates tumor formation and metastasis by systematic comparison of the mRNA profiles in CD90+ esophageal TICs and their differentiated progenies. Our findings suggest that ESCC metastasis is dictated, at least in part, through an altered Ets-1 transcription and downstream targets matrix metalloproteinase (MMP) and a deregulated epithelial–mesenchymal transition (EMT) phenotype. Discovery of this novel tumorigenic and metastatic TIC subpopulation in ESCCs provides an appropriate target cell for development of effective therapeutic strategies for ESCCs.

Materials and Methods

Fresh clinical tissue specimen collection and processing

Fresh human esophageal tumor and adjacent nontumor tissue specimens were obtained in accordance with the ethical standards of the Institutional Committee on human experimentation from 8 patients undergoing esophageal resection for ESCC. Specimens were collected between 2011 and 2012 at the Queen Mary Hospital in Hong Kong. Histologic examination was carried out by pathologists, and diagnosis was made based on the microscopic features of the carcinoma cells. Surgical specimens were obtained at the time of resection from all patients. All samples used in this study were approved by the committee for ethical review of research involving human subjects at Zhengzhou University (Zhengzhou, China).

Human esophageal cell lines

ESCC cell lines EC18 and EC109 were provided as a gift by Professor George Tsao (Department of Anatomy, The University of Hong Kong, Hong Kong, China). ESCC cell lines HKEsCl and KYSE520 were kindly provided by Professor Gopesh Srivastava (Department of Pathology, The University of Hong Kong). ESCC cell lines KYSE30, KYSE140, KYSE180, KYSE410, and KYSE510 were obtained from DSMZ, the German Resource Centre for Biological Material (8). All cell lines used in this study were regularly authenticated by morphologic observation and tested for absence of mycoplasma contamination (MycopAlert, Lonza).

Flow cytometry and cell sorting

Flow cytometric analysis or flow cytometric cell sorting was conducted using phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD90 (BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human CD271 (p75NTR; Miltenyi Biotec GmbH), and FITC-conjugated monoclonal mouse anti-human CD44 (Miltenyi Biotec GmbH). Samples were analyzed and sorted on BD FACSCanto II and FACS Aria I, respectively (BD Biosciences) with data analyzed using FlowJo software (Tree Star Inc.). For analysis and cell sorting of freshly resected clinical samples and xenograft tumors, cells would also be stained with 7-aminoactinomycin D (AAD; BD Biosciences) to exclude dead cells. Similar flow cytometry and cell sorting protocols have also been described in our previous studies (9, 10).

RNA extraction, cDNA synthesis, and real-time qPCR

Total RNA was isolated using the TRIzol Reagent (Invitrogen). cDNA was synthesized using the Advantage RT-for-PCR Kit (Clontech) and used for qPCR analysis. qPCR was carried out using SYBR Green PCR master mix (Applied Biosystems), and primers as listed in Supplementary Table S1. cDNA was amplified as an internal control. Experiments were carried out using an ABI Prism 7900 System (Applied Biosystems), and data processing was conducted using ABI SDS v2.1 software (Applied Biosystems).

Western blot

Protein lysates were resolved on SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore), and probed with mouse anti-human CD90 (BD Biosciences), vimentin (Cell Signaling), E-cadherin (Santa Cruz), Ets-1 (Santa Cruz), MMP2, MMP10 (Abcam), β-actin (Santa Cruz), and rabbit anti-human MMP13 (Abcam), followed by incubation with secondary horseradish peroxidase (HRP)-conjugated antibodies. The antibody signal was detected using an enhanced chemiluminescence system (Amersham Biosciences).
CD90 overexpression and knockdown

CD90 overexpression and knockdown plasmids were kindly provided by Professor Maria Lung and Dr. Hong-Lok Lung (Department of Clinical Oncology, The University of Hong Kong). Briefly, the full-length wild-type CD90 cDNA flanked with BamHI restriction sites was ligated to the pCR3.1 neomycin resistance–tagged expression plasmid. pCR3.1-CD90 recombinant and control pCR3.1 (Invitrogen) vector-alone plasmids were transfected into KYSE180 and EC109 cells with Lipofectamine 2000 Reagent (Invitrogen). Stable clones were selected using neomycin (11). For CD90 knockdown, 3 pairs of CD90 short hairpin RNA (shRNA) oligonucleotides were designed by BLOCK-iT RNAi designer program, as previously described (12). These oligonucleotides target positions 78–99, 298–117, and 301–321 of the human CD90 cDNA (NM_006288). shRNA oligonucleotides were ligated into a linearized pENTR/H1/TO plasmid and transfected into KYSE140 cells with Lipofectamine 2000 Reagent (Invitrogen). CD90 recombinant and control pCR3.1 (Invitrogen) vectors target positions 78–99, 298–117, and 301–321 of the full-length wild-type CD90 cDNA (NM_006288). Oligonucleotide microarray analysis

Oligonucleotide microarray analysis

Altered gene expression patterns of CD90+ and CD90− cells isolated from ESCC cell line KYSE140 were compared using the Affymetrix human genome U133 Plus 2.0 GeneChip (Affymetrix), covering 47,000 transcripts and variants. Experiments were carried out as a service at the Genome Research Centre of The University of Hong Kong. Scanned output files were analyzed using MicroArray Suite 5 (MAS5) method with Genespring GX (Affymetrix) and Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software. Transcripts with more than a 3-fold difference in expression level were defined as differentially expressed. Microarray data are available publicly at http://www.ncbi.nlm.nih.gov/geo/ (GEO accession number, GSE37949).

Sphere formation assay

Single cells were cultured in 300μL of serum-free DMEM:F12 medium (Invitrogen), supplemented with 20 ng/mL human recombinant EGF (Sigma-Aldrich), 10 ng/mL human recombinant basic fibroblast growth factor (bFGF; Invitrogen), 4 μg/mL insulin (Sigma-Aldrich), B27 (1:50; Invitrogen), 500 units/mL penicillin (Invitrogen), and 500 μg/mL streptomycin (Invitrogen). Cells were cultured in suspension in poly-HEMA–coated 24-well plates. Cells were replenished with 30μL of supplemented medium every second day. To propagate spheres in vitro, spheres were collected by gentle centrifugation and were dissociated to single cells using TrypLE Express (Invitrogen). Following dissociation, trypsin inhibitor (Invitrogen) was used to neutralize the reaction, and cells were cultured to generate spheres of the next generation. All cell culture was carried out at 37°C in a 5% CO2 humidified incubator.

Differentiation assay

For epithelial in vitro differentiation of stem cell–like cells, all-trans retinoic acid (ATRA), cis-diammine-platinum(II) dichloride (DPP; Sigma-Aldrich) was used to treat sorted CD90+ and CD90− cells at a concentration of 20 μmol/L in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin for 5 days, with medium change daily. Change in CD90 expression following treatment was assessed by flow cytometric analysis.

Chemoresistance assay

Cells were treated with various concentrations of 5-fluorouracil (5-FU) and taxotere (KYSE140 with 2.5 μg/mL 5-FU or 1 μg/mL Taxotere and KYSE520 with 10 μg/mL 5-FU or 2 μg/mL taxotere) for 48 hours. Cells would then be subsequently harvested and stained in binding buffer, propidium iodide (PI), and FITC-conjugated Annexin-V as provided by the Annexin-V FLUOS Staining Kit (Roche Diagnostics) according to manufacturer’s instructions. Analysis was determined by a FACSCanto II (BD Biosciences) and FlowJo software (Tree Star Inc.).

Tumorigenicity and serial transplantation assay in NOD/SCID mice

The study protocol was approved by and conducted in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong. For CD90 transplantation studies, CD90-sorted ESCC cells were injected subcutaneously into the flank of 4- to 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice in a mixture of complete medium and Matrigel in a 1:1 ratio. Each group contained 8 animals. Cryosections (5-μm thick) were stained with hematoxylin and eosin (H&E). Animals that were injected with tumor cells but showed no sign of tumor burden were generally terminated 5 months after tumor cell inoculation, and animals were opened up at the injection sites to confirm that there was no tumor development.

Cell invasion and motility assays

Invasion and migration assays were conducted in 24-well BioCoat Matrigel Invasion Chambers (BD Biosciences) according to manufacturer’s instructions or a 24-well millicell hanging insert (Millipore). In brief, 3 × 105 cells were added to the top chamber and 10% FBS in DMEM was added to the bottom chamber as a chemoattractant. After 48-hour incubation at 37°C, the number of cells that invaded through the Matrigel (invasion) or membrane (migration) was counted in 10 fields under a ×20 objective lens and imaged using SPOT imaging software (Nikon).

Experimental metastasis assay in NOD/SCID mice

Five-week-old NOD/SCID mice were used, and each experimental group consisted of 6 mice. Briefly, 3 × 105 cells were injected intravenously through the tail vein into each mouse. All mice were euthanized 20 weeks after injection. The livers and lungs were excised and embedded in paraffin. The presence of tumor nodules in the lung and liver was microscopically determined following H&E staining. The study protocol was approved by and conducted in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong.

Oligonucleotide microarray analysis

Altered gene expression patterns of CD90+ and CD90− cells isolated from ESCC cell line KYSE140 were compared using the Affymetrix human genome U133 Plus 2.0 GeneChip (Affymetrix), covering 47,000 transcripts and variants. Experiments were carried out as a service at the Genome Research Centre of The University of Hong Kong. Scanned output files were analyzed using MicroArray Suite 5 (MAS5) method with Genespring GX (Affymetrix) and Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software. Transcripts with more than a 3-fold difference in expression level were defined as differentially expressed. Microarray data are available publicly at http://www.ncbi.nlm.nih.gov/geo/ (GEO accession number, GSE37949).
Statistical analysis

All statistical analyses were conducted using PASW Statistics 18.0 (SPSS Inc.), with the exception of the significance in bar graphs, in which case analyses were conducted by applying the independent t test using Microsoft Office Excel software (Microsoft Corp.). Differences in CD90 expression among different clinicopathologic stages were analyzed by \( \chi^2 \) test. CD90\text{high} and CD90\text{low} in the qPCR data set is defined as patients showing higher or lower CD90 expression values than the median CD90 expression value. \( P < 0.05 \) was considered significant.

Results

CD90 expression in ESCC clinical specimens and cell lines

We have previously conducted RNA-Seq on 3 pairs of matched tumor and adjacent nontumor (NT) tissues from patients with ESCCs of Chinese origin using Illumina Genome Analyzer IIx platform (Solexa) and identified a number of differentially expressed genes (13). Among these genes, CD90 was found to be commonly upregulated in all 3 patient ESCC samples compared with its corresponding NT counterparts (patients #16, 18, and 19; Fig. 1A). Expression of other cancer stem cell–associated markers that have previously been reported to play a role in ESCCs (i.e., CD44, p75NTR/CD271, ABCG2, and BMI1) were also likewise detected, with the exception of CD133, in which case its expression was absent in all 3 matched samples (Supplementary Table S2; refs. 5–7, 14, 15). CD90 was a candidate gene of particular interest as it was the only moderately but statistically significantly upregulated surface glycoprotein (Supplementary Table S2), and also previously implicated to mark TICs of other organ types including hepatocellular carcinoma, glioblastoma, and T-acute lymphoblastic leukemia (16–18). Real-time quantitative PCR was carried out to validate this finding and results were concordant with the RNA-Seq results (Fig. 1B). To determine whether upregulation of CD90 was a common event in ESCCs, we extended our analysis to an additional 33 paired NT/primary ESCC samples. By qPCR, CD90 was found to be significantly upregulated in a majority of primary ESCC (81.8%, 27 of 33) when compared with their corresponding NT esophageal tissue samples (Fig. 1C). CD90 expression in ESCC also correlated with a stronger family history of ESCC (\( P < 0.019 \)) and higher incidences of lymph node metastasis (\( P < 0.049; \) Table 1).

In addition, we also examined the expression of CD90 in ESCC cell lines (\( n = 9 \)) and in fresh tissue samples derived from patients with ESCC (\( n = 8 \)) using flow cytometry. A small CD90\text{−} subpopulation was observed in all ESCC specimens (2.26%–10.10%; \( n = 8 \)) and the majority of ESCC cell lines (8 of 9) examined (Fig. 1D and E, Table 2 and Supplementary Table S3). In contrast, CD90 expression was either absent or detected at very low levels in their nontumor (0.51%–1.44%; \( n = 6 \)) and dysplastic (0.84%; \( n = 1 \)) counterparts (Fig. 1E and Table 2). Although not statistically significant due to our limited sample size, it is also worthy to note that the only patients with ESCCs with tumor recurrence (patient #8) displayed the highest CD90 expression levels of all the 8 freshly resected clinical samples examined (Table 2).

CD90\text{−} ESCC cells possess both cancer and stem cell–like properties

Next, we evaluated the tumorigenic potential and stem cell–like properties of isolated CD90\text{−} cells from ESCC cell lines KYSE140 and KYSE520. We first examined the ability of the cells to grow as spheroids in nonadherent, serum-free, growth factor–supplemented conditions that favor the proliferation of undifferentiated cells. Within 3 weeks of culture, we obtained ESCC spheres of growing undifferentiated CD90\text{−} cells. CD90\text{−} tumor cells invariably died in such serum-free conditions (Fig. 2A). More importantly, single cells obtained from these CD90\text{−}–dissociated spheres could be clonally expanded in subsequent serial propagations, showing virtually unlimited growth potential. Next, we investigated the ability of sorted cells to subcutaneously engraft and give rise to tumors in immunodeficient NOD/SCID mice. As few as 5 \( \times 10^4 \) CD90\text{−} cells resuspended in Matrigel generated visible tumors 12 weeks postinjection, whereas, in contrast, as many as 3.2 \( \times 10^5 \) CD90\text{−} ESCC cells still failed to initiate tumor formation 24 weeks postinjection (Fig. 2B and Table 3). Histologic analysis revealed that tumor xenografts generated indeed displayed an ESCC phenotype (Fig. 2B). These data indicate that cells capable of initiating ESCCs are highly enriched within a CD90\text{−} cell population. To investigate whether CD90\text{−} ESCC cells display long-term tumorigenic potential and self-renewing capacity, we evaluated their ability to generate tumors in serial transplantations. Sorted CD90\text{−} and CD90\text{+} cells from primary tumor xenografts were transplanted into secondary mouse recipients. Only CD90\text{−} tumor cells successfully engrafted with the exception of one successful tumor xenograft by CD90\text{−} cells in one mouse. CD90\text{−} cells in the secondary passage also grew better than CD90\text{+} counterparts, as evident by the number of tumor-bearing mice and tumor-initiating frequency (Table 3).

To determine the differentiation potential of these CD90\text{−} cells, sorted CD90 cells were cultivated in the presence of the differentiation inducer, retinoic acid (ATRA) at a concentration of 20 \( \mu \)mol/L. As compared with CD90\text{−} counterparts and or dimethyl sulfoxide (DMSO) negative controls, CD90\text{−} cells expressed a greater difference in CD90 expression following induced in vitro differentiation. Expression of CD90 was significantly downregulated in ATRA-induced differentiated CD90\text{−} cells (from 99% to 72% in KYSE140 and from 97.9% to 30.4% in KYSE520) as compared with CD90\text{−} cells treated with DMSO controls (from 99% to 88.3% in KYSE140 and from 97.9% to 39.7% in KYSE520). CD90\text{−} cells treated with ATRA displayed a significantly smaller increase in CD90 expression, as compared with the decrease of CD90 expression in CD90\text{−} cells, showing that CD90\text{−} cells indeed possess a greater differentiation potential than CD90\text{−} cells (Fig. 2C). And finally, to investigate whether CD90\text{−} subpopulation displayed enhanced chemoresistant potential, sorted CD90 cells were treated with chemotherapeutic reagents 5-FU or taxotere in vitro and analyzed for apoptotic/necrotic cells by flow cytometry as well as for CD90\text{−} subsets by real-time qPCR. CD90\text{−} cells were found to be more viable than corresponding CD90\text{−} counterparts, as evident by the lower apoptotic rates. 5-FU- and taxotere-induced apoptosis increased close to 2-fold in CD90\text{−} as opposed to CD90\text{−} cells (Fig. 2D). Furthermore,
treatment of unsorted ESCC cell lines KYSE140, KYSE520, and HKESC1 with either 5-FU or taxotere led to significant enrichment of CD90<sup>+</sup> subpopulations (Fig. 2E). In addition, CD90<sup>+</sup> cells isolated from KYSE140 and KYSE520 also expressed an enhanced level of the stem cell–associated gene, nestin (Fig. 3A). Collectively, these data show the existence of a TIC population in ESCCs marked by a CD90 surface phenotype and bearing cancer and stem cell–like features including the ability to self-renew, differentiate, initiate tumors in vivo, and resist standard chemotherapy.

mRNA profiling of CD90<sup>+</sup> esophageal TICs and CD90<sup>−</sup> cell counterparts identifies aggressive gene expression signatures in CD90<sup>−</sup> TICs

In an attempt to characterize the molecular mechanisms by which CD90<sup>+</sup> esophageal TICs mediate tumor formation and growth, a genome-wide mRNA expression profiling screen was subsequently used to compare gene expression profiles between CD90<sup>−</sup> esophageal TICs and their CD90<sup>−</sup> counterparts isolated from ESCC cell line KYSE140. Using a stringent fold change of 3, 154 differentially expressed genes were
Identified, including 92 upregulated genes and 62 downregulated genes (Supplementary Table S5). Further analysis by IPA software identified a significantly altered functional network involving the transcription factor Ets-1 and downstream target matrix metalloproteinase, including MMP1, 2, 10, and 13 (Supplementary Fig. S1). Deregulation of these genes was subsequently validated at both genomic and proteomic levels in sorted CD90 subpopulations isolated from ESCC cell lines KYSE140, KYSE520 as well as ESCC clinical sample (HKU ESCC), by real-time qPCR and Western blotting (Fig. 3B). Given that both Ets-1 and different MMPs have previously been reported to be overexpressed in ESCCs (19–21) and that Ets-1 induction of MMPs has also previously been found to orchestrate the malignant phenotype of different cancer types (22, 23), we hypothesize whether CD90+ esophageal TICs also possess metastatic potential.

**CD90**+ esophageal TICs possess metastatic potential in vivo

To test whether CD90+ esophageal TICs displayed enhanced invasive and migratory properties as compared to CD90− cells, Matrigel invasion and Transwell migration assays were conducted. CD90+ was found to possess an enhanced ability to both invade and migrate (Fig. 3C and D). To further confirm this observation, an experimental tail vein metastasis model was also conducted in vivo. CD90+ esophageal TICs, but not its CD90− counterparts, displayed a superior ability to metastasize to the lung following cell injection through the tail vein (Fig. 3E). Metastasis of tumor cells is believed to involve the loss of cell–cell interaction together with the acquisition of migratory properties and is often associated with EMT of cells. Thus, we also evaluated the expression of E-cadherin and vimentin, both hallmarks of the EMT phenotype. Expression of the epithelial marker E-cadherin was found downregulated in CD90+ TICs, whereas the mesenchymal marker vimentin was found upregulated in CD90+ TICs as compared with CD90− cells (Fig. 3B). Taken together, these findings suggest that CD90+ esophageal TICs contributes to the invasive phenotype and metastatic capacity of ESCCs through a deregulated Ets-1 induced MMP signaling and an altered EMT phenotype.

To explore the functional role of CD90 in ESCCs, we also overexpressed CD90 in ESCC cell lines KYSE180 and EC109 and repressed CD90 in KYSE140 and assessed its potential to self-renew, initiate tumor formation, invade, and migrate in vitro and in vivo. Cells with or without CD90 expression manipulated showed a similar ability to form spheroid, initiate tumor growth in immunodeficient mice as well as to invade and

---

Table 1. Clinicopathologic correlation of CD90 expression in ESCC

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD90low (n=22)</th>
<th>CD90high (n=14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5 (29.4%)</td>
<td>7 (43.8%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (70.6%)</td>
<td>9 (56.3%)</td>
<td>0.392</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>7 (41.2%)</td>
<td>10 (62.5%)</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>10 (58.8%)</td>
<td>6 (37.5%)</td>
<td>0.221</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (31.2%)</td>
<td>11 (73.3%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>11 (68.8%)</td>
<td>4 (26.7%)</td>
<td>0.019a</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (17.6%)</td>
<td>8 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14 (82.4%)</td>
<td>8 (50.0%)</td>
<td>0.049a</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>1 (5.9%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>5 (29.4%)</td>
<td>1 (6.3%)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>11 (64.7%)</td>
<td>15 (83.8%)</td>
<td>0.119</td>
</tr>
<tr>
<td>Tumor size, cm3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥12</td>
<td>9 (52.9%)</td>
<td>8 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>&lt;12</td>
<td>8 (47.1%)</td>
<td>8 (50.0%)</td>
<td>0.866</td>
</tr>
</tbody>
</table>

*P value < 0.05.

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Table 2. Clinicopathologic variables and flow cytometric analysis of CD90 expression in freshly resected nontumor, dysplastic, and ESCC clinical samples

<table>
<thead>
<tr>
<th>Patient's clinicopathologic information</th>
<th>CD90 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Sex TM stage Differentiation Treatment Recurrence Other information Nontumor Dysplastic ESCC</td>
<td></td>
</tr>
<tr>
<td>#2 M T4N2M0 Poor R N</td>
<td>—</td>
</tr>
<tr>
<td>#3 M T0N0M0 N/A CTRT + R N</td>
<td>0.97%</td>
</tr>
<tr>
<td>#4 M T3N2M0 Poor R N</td>
<td>0.51%</td>
</tr>
<tr>
<td>#5 M T3N2M0 Moderate R N</td>
<td>0.51%</td>
</tr>
<tr>
<td>#6 M T1BN1M0 Moderate CTRT + R N</td>
<td>—</td>
</tr>
<tr>
<td>#7 M T1BN1M0 Moderate/poor R N</td>
<td>1.12%</td>
</tr>
<tr>
<td>#8 M T3N0M0 Poor R Y</td>
<td>0.68%</td>
</tr>
<tr>
<td>#9 M T3N0M0 Poor R N</td>
<td>1.44%</td>
</tr>
<tr>
<td></td>
<td>0.85%</td>
</tr>
</tbody>
</table>

Abbreviations: —, no sample collected; CTRT + R, chemoradiation treatment and resection; N/A, information not available; R, resection.
migrate. These cells also showed similar expression of Ets-1, vimentin, E-cadherin, and MMPs, thus suggesting that CD90 may serve as only a marker but have no specific functional role in ESCCs (data not shown).

Overlapping expression with CD271 and CD44

To examine whether our CD90⁺ esophageal TIC subpopulation display overlapping expression with other known esophageal TIC markers, we first conducted single-color flow cytometry.
To our knowledge, this is the first study to identify TIC cells from freshly resected clinical samples and also the first study to

cytometric analysis on a panel of ESCC cell lines to detect CD90, CD44, and/or p75NTR (CD271) expression separately (Supplementary Table S3), and then on the basis of these results, we subsequently conducted dual-color flow cytometric analysis on selected ESCC cell lines and a clinical sample to detect for overlapping expression of CD90 with CD44 or p75NTR (Fig. 4). CD90 expression displayed only a small overlapping expression with both CD44 and p75NTR in KYSE520 (10.4% CD44+CD90+ and 6.65% p75NTR+CD90+) and ESCC clinical sample patient #8 (0.75% CD44+CD90+ and 11.4% p75NTR+CD90+). Isolated SP cells showed greater clone formation efficiency in vitro and tumor formation ability in vivo (24). Isolated SP cells showed greater clone formation efficiency in vitro and tumor formation ability in vivo (24). Isolated SP cells showed greater clone formation efficiency in vitro and tumor formation ability in vivo (24). Isolated SP cells showed greater clone formation efficiency in vitro and tumor formation ability in vivo (24).

### Table 3. In vivo tumor development and serial transplantation experiments of CD90+ and CD90− subpopulations isolated from ESCC cells KYSE140 and KYSE520

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell number</th>
<th>Tumor incidence</th>
<th>Cell number</th>
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<td>1/2</td>
<td>5,000</td>
<td>1/5</td>
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<tr>
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<td>2/2</td>
<td>10,000</td>
<td>2/5</td>
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<tr>
<td></td>
<td>320,000</td>
<td>2/2</td>
<td>50,000</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>Tumor-initiating frequency: 1 in 10,345</td>
<td>Tumor-initiating frequency: 1 in 108,980</td>
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<tr>
<td>KYSE520 CD90−</td>
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<td>—</td>
<td>Groups as above</td>
<td>—</td>
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<td>Tumor-initiating frequency: 1 in 57,076</td>
<td>Tumor-initiating frequency: 1 in 1,000,626</td>
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<tr>
<td>KYSE520 CD90+</td>
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and CD271 combined was not indicative of a better prognostic marker of ESCCs.

### Discussion

To date, the TIC hypothesis has been confirmed in a wide variety of solid tumors, including breast, brain, colon, head and neck, prostate, liver cancers, etc. (reviewed in ref. 4). However, only a few studies have focused on ESCCs, with experiments limited to only the use of cell lines. TIC-like cells in the form of side population (SP) marked by Hoechst 33342 dye were first isolated from ESCC cell lines EC9706 and EC109 by Huang and colleagues in (24). Isolated SP cells showed greater clone formation efficiency in vitro and tumor formation ability in vivo (CD271), a stem cell marker for normal human esophagus, was also studied in ESCC cell lines by 2 independent groups led by Huang and colleagues and Okumura and colleagues (6, 7). p75NTR-positive cells were found to possess a greater ability to form colonies in vitro (7) as well as to self-renew and resist chemotherapy (6). However, these studies were again limited as they were only focused on KYSE (7) or Eca109, SHEC-1, SHEC-4, and SHEC-5 ESCC cell lines (6). More recently, Zhang and colleagues also found tumor spheres of Eca109, SHEC-1, SHEC-4, and SHEC-5 ESCC cell lines (6). However, the ALDH1 isoform was used for CD90 and CD44 or CD90 and CD90 and CD75NTR) was needed to distinguish the metastatic subset of esophageal TICs. We also carried out qPCR to examine expression of CD44 and p75NTR/CD271 and its clinicopathologic significance on the same set of paired nontumor and ESCC clinical samples (n = 33) used for CD90 analysis (Fig. 1C). CD44 was consistently detected at a higher expression level in the majority of ESCCs with reduced levels in the nontumor sectors (paired t test; P < 0.001; Supplementary Fig. S2). Expression of CD271 was not found to be significantly different in nontumor and ESCC samples (paired t test; P = 0.384; Supplementary Fig. S2). No significant association was observed between CD44 and CD271 with clinicopathologic variables (Supplementary Table S4). Elevated expression of CD90 and CD44 combined or CD90
identify an esophageal TIC subset within primary tumors that harbor both tumor-propagating and metastatic capacity. By transcriptome sequencing, we found CD90 to be commonly upregulated (at moderate levels as indicated by their low RPKM values) in all 3 ESCC clinical samples as compared with their respective nontumor counterparts. Note that our RNA-Seq profiling was also able to pick up other cancer stem cell–associated markers that have previously been reported to play...
a role in ESCCs (i.e., CD44, p75NTR/CD271, ABCG2, and BMI1; Supplementary Table S2; refs. 5–7, 14, 15). Although these genes were also likewise found to be upregulated in ESCCs as compared with its nontumor counterparts, their expression was either detected in ample amounts (i.e., CD44 with high RPKM values: range, 8–90) or conversely at really low expression levels (i.e., p75NTR, ABCG2, and BMI1 with very low RPKM values: range, 0–3). On the basis of the 3 paired NT and ESCC samples sent for RNA-Seq profiling (patients #16, #18, and #19), only CD90 and CD44 was found to have a fold change that was statistically significant (5.02E-07 for CD90 and 2.41E-04 for CD44). CD133 had no expression in all but sample 18T where only one exon read could be detected. As the P value of CD90 fold change was of highest significance, CD90 was subsequently chosen for further study. By both qPCR and flow cytometric analyses in a larger cohort of clinical samples, we subsequently validated our RNA-Seq observation and confirmed that CD90 is indeed upregulated in ESCCs. Of more interest is that we also found CD90 expression in ESCCs to be associated with a strong family history of ESCCs, which possibly indicates the usefulness of CD90 expression to predict ESCC incidences in families where ESCCs is prevalent. Furthermore, CD90 expression in ESCCs was also found to be tightly correlated with lymph node metastasis, which further lends support to our observations in the second part of our study where we found CD90 to also mark the metastatic TIC subset of ESCCs. CD90, also known as Thy-1, has previously been found to mark TIC subpopulations of hepatocellular carcinoma (16), glioma (17), and T-acute lymphoblastic leukemia (18). CD90+ cells in these cancers were formerly found to exhibit classic cancer and stem cell-like features. Likewise, in ESCCs, we also found CD90+ cells to display an enhanced ability to self-renew, initiate tumor in vivo, differentiate, and resist chemotherapy. In addition, our study is also the first to identify a metastatic TIC subpopulation in ESCC. CD90+ TICs were found to exhibit enhanced invasive and migratory abilities as shown both in vitro and in vivo. Subsequent microarray analysis found CD90+ TICs to exhibit an enhanced expression of aggressive gene signatures including higher expression of the transcription factor Ets-1, its downstream targets, MMP2, MMP10, MMP13, as well as classic EMT phenotypes including increased expression of mesenchymal marker vimentin and decreased expression of epithelial marker E-cadherin. Our observations are consistent with previous publications by other groups where Ets-1, MMP expression, and activation were also found to correlate with the malignant phenotype of ESCCs (19–21). Findings in the second part of our study further layer another level of complexity to this knowledge where we now find an enhanced Ets-1, MMP, and EMT phenotype in the specific subpopulation of TICs in ESCC marked with CD90+ surface phenotype. As previously mentioned, CD44 and p75NTR (CD271) have both been previously reported to mark the tumorigenic TIC subset of ESCCs. Here, dual-color flow cytometric analysis of CD44 and CD90 or p75NTR and CD90 found only a small overlap between these markers, possibly suggesting that (i) CD90 in combination with CD44 or p75NTR can better discriminate the tumorigenic/metastatic TIC subset of ESCCs or (ii) there exists multiple TIC pools within ESCC tumors that can be distinguished by CD44, p75NTR, or CD90.

Figure 4. Dual-color flow cytometric analyses of CD90 with CD44 (top) and CD90 with p75NTR (CD271; bottom) in ESCC cell lines (KYSE140 and KYSE520) and ESCC clinical specimen (patient #8).
and characterization of these cells. The identification and characterization of tumorigenic and metastatic esophageal TICs and the molecular targets downstream of these cells in this study will hopefully provide insight into the gradual improvement of more effective cancer therapies against this deadly disease. Further characterization of the molecular mechanism by which CD90+ TICs drives tumorigenesis and metastasis in ESCCs is warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.H. Tang, Y. Dai, S.W. Tsao, S. Ma, X.-Y. Guan
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.H. Tang, Y. Dai, S.V. Tong, P.S. Kwan, Y.Q. Qin, S.W. Tsao, M.L. Lung, D.K. Tong, S. Law, K.W. Chan, S. Ma, X.-Y. Guan
Analysis and interpretation of data (e.g., statistical analysis, biosistics, computational analysis): K.H. Tang, Y. Dai, Y.-P. Chan, S. Ma
Writing, review, and/or revision of the manuscript: K.H. Tang, S. Ma

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Tong, L. Fu, H.L. Lang, S. Law, K.W. Chan, X.-Y. Guan
Study supervision: S. Ma, X.-Y. Guan

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Kwan Ho Tang, Yong Dong Dai, Man Tong, et al.


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