

Original Research

Single-Cell Transcriptome Analysis of Small Cell Neuroendocrine Carcinoma of the Endometrium Reveals *ISL1* as a Potential Biomarker for Diagnosis and Treatment

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Abstract

Background: As a dedifferentiated tumor, small cell endometrial neuroendocrine tumors (NETs) are rare and frequently diagnosed at an advanced stage with a poor prognosis. Current treatment recommendations are often extrapolated from histologically similar tumors in other sites or based on retrospective studies. The exploration for diagnostic and therapeutic markers in small cell NETs is of great significance. Methods: In this study, we conducted single-cell RNA sequencing on a specimen obtained from a patient diagnosed with small cell endometrial neuroendocrine carcinoma (SCNEC) based on pathology. We revealed the cell map and intratumoral heterogeneity of the cancer cells through data analysis. Further, we validated the function of ISL LIM Homeobox 1 *(ISL1) in vitro* in an established neuroendocrine cell line. Finally, we examined the association between *ISL1* and tumor staging in small cell lung cancer (SCLC) patient samples. Results: We observed the significant upregulation of *ISL1* expression in tumor cells that showed high expression of the neuroepithelial markers. Additionally, *in vitro* cell function experiments demonstrated that the high *ISL1* expression group exhibited markedly higher cell proliferation and migration abilities compared to the low expression group. Finally, we showed that the expression level of *ISL1* was correlated with SCLC stages. Conclusions: *ISL1* protein in NETs shows promise as a potential biomarker for diagnosis and treatment.

Keywords: uterine neuroendocrine tumors; single-cell RNA sequencing; ISL1; cell proliferation and migration

1. Introduction

For more than a century, the unique morphological and clinical features of neuroendocrine tumors (NETs) have attracted much attention of surgeons, pathologists and doctors. NETs are rare tumors that can develop in almost every organ and tissue in the human body. Even though the hormones secreted and the origin site are different, NETs in different organs are similar [1]. Poorly differentiated epithelial tumors are defined as neuroendocrine carcinomas (NECs), which comprise cells with severely deranged molecular/genetic characteristics and severe cellular atypia but widely retain neuroendocrine markers [2]. NECs are of high grade by default and are classified into large cell NEC (LCNEC) and small cell NEC (SCNEC).

Endometrial cancer (EC) is the most common gynecological cancer in high-income countries. In addition, its incidence rate is increasing worldwide [3]. Postmenopausal bleeding is a common early manifestation of EC and can be treated by hysterectomy; however, patients with advanced disease have a poor prognosis. It is very important that women adopt individualized treatment to provide primary prevention for those at high risk and improve the survival rate and prognosis of patients with EC. Minimally invasive surgical staging and some new technologies such as sentinel lymph node biopsy are alternatives for surgical treatment and do not affect the oncological results [4]. Adjuvant radiotherapy can reduce local recurrence in moderate and high-risk cases. Advances in the molecular genetics of EC have paved the way for targeted chemotherapy strategies [5,6]. All treatment plans and prognoses are closely related to the pathological classification of EC. The World Health Organization classifies EC according to its morphology [7]. The most common type of EC is endometrioid carcinoma, whereas serous, clear cell, undifferentiated, and dedifferentiated carcinomas are less commonly observed.

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As a type of dedifferentiated cancer, uterine NETs are rare, accounting for about 1% of all ECs. Most women have abnormal vaginal bleeding or symptomatic metastatic disease. Like other EC histologies, it can be diagnosed through endometrial biopsy or curettage. Women with endometrial NETs typically present with advanced disease (stage III and stage IV) in 55.7% of cases [8]. NETs of the gynecologic tract are rare, so treatment guidelines are limited. Current treatment recommendations are usually inferred from tumors with similar histology in other organs, or based on retrospective studies [9].

Single cell RNA sequencing (scRNA-seq) is a technique for analyzing complex tissue transcriptome at the single-cell level. This technology can help identify differential gene expression and epigenetic factors which are caused by single-cell genome mutations. scRNA-seq are playing a very significant role in all aspects of tumor research. It not only reveals the heterogeneity of tumor cells, but also monitors tumor progression, and prevents further cell deterioration. In addition, transcriptome sequencing analysis of corresponding immune cells in tumor tissue can be used to classify immune cells, which can help analyze the immune escape and drug resistance mechanisms of tumors, and provide effective clinical targeted therapies [10]. scRNA-seq provides a powerful new way to describe clone diversity and understand the role of rare cells in the development of EC.

In this study, we performed single-cell sequencing in a patient diagnosed with endometrial SCNEC. The results revealed the complexity of the cell composition of SC-NEC tumor cells and stromal cells, and provide meaningful biomarkers for SCNEC development and progression based on intratumoral heterogeneity analysis, which were verified in an NET cell line.

2. Materials and Methods

2.1 Sources of the Human Tissue Samples and Cell Lines

The human tissue samples from small cell neuroendocrine carcinoma of the endometrium involved in this study were from Clinical and Translational Research Center of Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, 200120, China. The section samples of small cell lung cancer were from patients who underwent pulmonary surgery at Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, 200433, China. The human NCI cell lines H446 used in this study was from National Collection of Authenticated Cell Cultures. This study was approved by the Clinical Research Ethics Committee of the College of Medicine, Tongji University.

2.2 Histopathology and Immunohistochemistry

Tissue samples were collected, fixed and then paraffin-embedded. 5 μ m sections were obtained and subjected to histopathology and immunohistochemistry (IHC)

analysis. We used Hematoxylin and eosin (HE) staining for histopathological examination and immunohistochemistry for expression analysis, respectively. The "UltraVision Quanto Detection System HRP DAB" IHC Kit (TL-125-QDH; Thermo Fisher Scientific, Waltham, MA, USA) was used for IHC assay according to the manufacturer's protocol. In this study, the primary antibodies used were as follows: anti-cytokeratin pan (GM351529; Gene Tech, Shanghai, China), anti-cluster of differentiation 56 (CD56) (Kit-0028; MXB Biotechnologies, Fuzhou, China), anti-synaptophysin (Kit-0022; MXB Biotechnologies), anti-chromogranin A (MAB-0707; MXB Biotechnologies), anti-Ki67 (IR626; Dako Products, Santa Clara, CA, USA), anti-vimentin (Y23037; Ventana Medical Systems, Oro Valley, AZ, USA), anti-CD3 (ab16669; Abcam, Cambridge, MA, USA), anti-CD20 (M0755; Dako Products), and anti-programmed death-ligand 1 (ab205921; Abcam). Images were taken using a digital pathology scanner (Pannoramic MIDI II; 3DHISTECH Ltd., Budapest, Hungary).

2.3 Preparation of a Single-Cell Suspension, $10 \times$ Library Preparation and Sequencing

The single-cell suspension was generated carefully and rapidly to ensure high viability as previously described [11]. Briefly, the tissue was initially subjected to mechanical dissociation and then enzymatic degradation with collagenase type I/II (Thermo Fisher Scientific) and DNAse I (Sigma, St. Louis, MO, USA). Following digestion, the cell suspension was subjected to erythrocytes removing by red blood cell lysis buffer (Solarbio Life Science, Beijing, China) and further live-cell enrichment with Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Hemocytometer and Countess cell counter were used to count cell number and examine cell viability, respectively. Finally, when the cell viability is bigger than 85%, cell suspension at a concentration of 1×10^6 cells/mL was eligible for subsequent $10 \times$ library preparation and sequencing.

The Chromium Single cell 3' Reagent v2 Kit ($10 \times$ Genomics, Pleasanton, CA, USA) and the Chromium Single Cell Controller Instrument were used to generated $10 \times$ single-cell library according to the manufacturer's instructions. After library construction quality control, sequence data were generated using Illumina HiSeq X Ten System (Illumina, San Diego, CA, USA).

2.4 Single-Cell Transcriptome Data Preprocessing and Analysis

We used the Cell Ranger software pipeline (version $3.1.0, 10 \times \text{Genomics}, \text{https://www.10xgenomics.com/sup}$ port/software/cell-ranger) to process the reads, including: mapping reads to the human reference genome (GRCh37) and transcriptome and generating a matrix of gene counts versus cells. Then, we used the Seurat (version 3.1.2, https://satijalab.org/seurat/) R package for downstream analy-



sis. Briefly, we removed low-quality cells by quality control and generated normalized count by "NormalizeData" function. Principal component analysis (PCA) was performed to reduce the dimensionality. Then we used the Seurat "Find Clusters" algorithm and "Run TSNE" function to obtain a graph-based clustering result visualized in 2-dimension. "Find Markers" function was used to find differentially expressed genes (marker genes, p < 0.05 and |log2foldchange| > 0.58) in each cell cluster. Cell types were identified using canonical marker genes.

2.5 Gene Ontology Enrichment Analysis

Gene Ontology (GO) enrichment analysis for differentially expressed genes (DEGs) was performed using R package based on the hypergeometric distribution. The z scores were computed from normalized –log10 (*p* value) generated from the Fisher exact test. GO enrichment graph was generated using R package pheatmap (version 1.0.12, https://www.rdocumentation.org/packages/pheatm ap/versions/1.0.12/topics/pheatmap).

2.6 Pseudotime Analysis

We performed dimensional reduction and clustering again to generate subclusters for original cluster 2 and cluster 9. Then, 8 subclusters were obtained, subclusters 1–5 were annotated as neuroendocrine tumor cells. The Monocle2 R package (version 2.9.0) [12] was used to perform the trajectory analysis on the tumor cells, so as to infer the relative differentiation time of each cell based on gene expression. Monocle2 R package functions were used to select ordering genes, reduce dimensional, and finally plot genes in pseudotime. The differentiation trajectory of tumor cells or the evolution of tumor cell subclusters during development can be deduced by pseudotime analysis.

2.7 Inferring Copy Number Variation Analysis

Initial copy number variations (CNVs) for each region were estimated using the inferCNV R package [13]. The CNV of total cell types was calculated by the expression level from single-cell sequencing data for each cell with a cutoff of 0.1. Genes were sorted based on their chromosomal location, and a moving average of gene expression was calculated using a window size of 101 genes. The expression was then centered to zero by subtracting the mean. The neuroepithelial (cluster 2) cells were selected as malignant cells, leaving all remaining cells as the normal cells. Denoising was carried out to generate the final CNV profiles. The relative CNV value on each chromosome in each cell was showed in the form of a heat map, and 5 CNV groups was clustered according to CNV level.

2.8 The Cancer Genome Atlas (TCGA) Data Download and Analysis

TCGA Uterine Corpus Endometrial Carcinoma (UCEC) patient RNA-seq data were downloaded

from the TCGA database project by UCSC Xena (https://xenabrowser.net/datapages/). The top and bottom 10% of patients based were selected on *ISL1* mRNA abundance followed with differential gene analysis with R package DESeq2 (version 1.42.0, https://bioconductor.org /packages/release/bioc/html/DESeq2.html).

2.9 Correlation Analysis

Spearman's correlation analysis was performed to assess the relationship between subclusters of single cell RNA-seq data and cancer cell line encyclopedia (CCLE) H446 RNA-seq data.

2.10 Cell Culture

The human NCI cell lines H446 which we used in the study was maintained in our laboratory from Chinese Academy of Sciences. H446 were validated by STR profiling and tested negative for mycoplasma. Then H446 cells were cultured in RPMI Medium 1640 with L-Glutamine (HyClone, Chicago, IL, USA) supplemented with 10% fetal bovine serum and 1% Pen/Strep (100 U/mL penicillin/100 μ g/mL streptomycin; Gibco, Carlsbad, CA, USA) under the conditions of 37 °C and 5% CO₂.

2.11 Cell Proliferation and Migration

H446 cells were seeded in 96-well plates $(3 \times 10^3 \text{ cells/well})$ for 24 h. The cell numbers were determined by using Cell Counting Kit-8 (CCK-8) at 450 nm with a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As for migration, H446 cells (8 × 10⁴ cells) were seeded into the upper chambers. After incubation for 18 h, each chamber was stained by using AM (calcein-acetoxymethyl ester, calcein-AM; Thermo Fisher Scientific) [14]. At last, the migrated cells were imaged by fluorescence analysis (Nikon, Tokyo, Japan).

2.12 Flow Cytometry

After cell counting, the cells were incubated with ISL LIM Homeobox 1 (*ISL1*) monoclonal antibody (1:200, 15661-1-AP; Proteintech, Rosemont, IL, USA) at 4 °C in the dark for 60 min, followed by incubation for 30 min with a fluorescent secondary antibody (1:200, SA00013-2, CoraLite488-conjugated goat anti-rabbit IgG (H+L)). The cells were washed twice with PBS at 4 °C (1500 rpm, 5 min) for testing. Finally, the positive cells were sterile sorted using the BD FACSAria high-speed flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.13 Statistical Analyses

Data were analyzed with GraphPad Prism (Version 9.5.1, https://www.graphpad.com/) software. For comparisons between two groups, statistical evaluation was done using the two-tailed Student's *t*-test. For multiple comparisons, two-way Analysis of Variance (ANOVA) test was used. The association between *ISL1* subtype and clinical



Fig. 1. Pathology details of the patient with endometrial small cell neuroendocrine carcinoma. (A) Diagnosis and treatment of the patient with endometrial small cell neuroendocrine carcinoma. (B) Hematoxylin and eosin (HE) staining and Pan-cytokeratin (CK-pan) expression detected by immunohistochemistry on formalin-fixed paraffin-embedded slides. Scale bars, 80 μm. (C) Expression of phenotype-related genes (*CD56, Synaptophysin, Chromogranin A*). Scale bars, 60 μm. (D–F) Expression of proliferation gene (*Ki67*), stromal cell markers (Vimentin, CD3, CD20) and immune checkpoint marker (Programmed death-ligand 1, PD-L1) detected by immunohistochemical analysis. Scale bars, 80 μm.

characteristics were explored by chi-square test. For all statistical tests, p < 0.05 was considered statistically significant. Error bars show the standard error of the mean.

3. Results

3.1 Single-Cell Expression Atlas in Small Cell Neuroendocrine Endometrial Carcinoma

A 60-year-old woman presented to our hospital with abnormal vaginal bleeding for more than 1 month. Pathological examination of endometrial curettage showed endometrial malignancy. Therefore, the patient underwent surgery, and pathological examination revealed high-grade SCNEC of the endometrium (Fig. 1A). IHC analysis of the sample showed as following: synaptophysin⁺⁺, CD56⁺, chromogranin A⁺, and Ki-67 80%, immune checkpoint marker Programmed death-ligand 1⁺ (PD-L1⁺). CD3 and CD20 staining showed immune infiltration in the tumor environment (Fig. 1B–F). Then we extracted tumor tissue for further single-cell sequencing.

The primary tumor sample was obtained after resection and subjected to single-cell RNA library preparation



Fig. 2. Single cell RNA sequencing (ScRNA-seq) profiling of tumor and stromal cells from the small cell endometrial neuroendocrine carcinoma (SCNEC) sample. (A) Experimental workflow of scRNA-seq procedure for the SCNEC tumor. (B) Quality control and metric information for the sequencing sample. (C) The remaining cells after quality control and filtering step. (D) The t-distributed stochastic neighbor embedding (t-SNE) projection where cells that share similar transcriptome profiles are grouped by colors representing unsupervised clustering results. (E) The t-SNE plot demonstrates the major cell types. (F) Expression of representative marker genes of the stromal cell types.

using the $10 \times$ Genomics Chromium platform (Fig. 2A). The mean reads per cell was 63,620, and the median number of genes detected per cell was 1248 (Fig. 2B). After quality filtering using the Seurat package, 10,214 cells remained for downstream analysis (Fig. 2C). Graph-based clustering revealed 12 clusters, and known marker genes were used to identify seven major cell types: neuroendocrine tumor cells (neurogenic differentiation 1 [NEUROD1] and neuronatin [NNAT]; cluster 2) [15], fibroblasts (type III collagen [COL3A1]; cluster 9), T cells (CD3 delta subunit of the T-cell receptor complex; clusters 1, 3 and 8), natural killer cells (granulysin [GNLY]; cluster 7), B cells (CD79A; clusters 5, 6, and 10), myeloid cells (CD68; clusters 4 and 11), and platelets [16] (triggering receptor expressed on myeloid cells-like [TREML1]; cluster 12) (Fig. 2D-F, Fig. 3E and Supplementary Fig. 1). IHC staining was performed to confirm the existence of stromal cell types such as fibroblasts, T cells, and B cells with vimentin, CD3, and CD20 antibodies (Fig. 1E). Overall, this analysis showed a complex cellular ecosystem in the SCNEC tissue.

3.2 Expression Heterogeneity and Cell Typing of Stromal and Cancer Cells

To decode the immune composition complexity, we further identified the cell subsets in lymphocytes and myeloid cells. We annotated two T cell subsets including conventional CD4⁺ T cells (CD4; cluster 1) and CD8⁺ T cells (CD8B; cluster 3) (Fig. 3A,B). Proliferative immune cells (marker of proliferation Ki-67 [MKI67]) consisted of both T and B cell lineages (Fig. 3B). We identified two subsets (clusters 4 and 11) of myeloid cells. Dendritic cells (cluster 11) and macrophages (cluster 4) were characterized by the enriched expression of CD1E and CD163, respectively (Fig. 3C). For B cells, follicular B cells (membrane spanning 4-domains A1 [MS4A1]; cluster 10) and plasma B cells (marginal zone B1 [MZB1]; clusters 5 and 6) were further revealed (Fig. 3D). Then we focused on the expression profile of the tumor cells. Differential gene expression analysis of each cluster revealed that cells in cluster 2 exhibited relatively high expression of neuroendocrine relevant genes, including NNAT, NEUROD1, nestin (NES), synaptophysin, and neural cell adhesion molecule 1 (Fig. 3E). These results generate a baseline tumor and stromal cell diversity atlas based on the single-cell NEC transcriptomes.

3.3 Intratumoral Heterogeneity of the Cancer Cells Reveals an ISL1-Positive Population

We identified tumor cell subclusters to explore their functions. The 1835 cells from clusters 2 and 9 were reclustered into eight distinct subclusters (Fig. 4A). Subclusters 6, 7, and 8 corresponded to endothelial cells (decorin [DCN]), fibroblasts (COL3A1), and epithelial cells (keratin 8 [KRT8] and epithelial cellular adhesion molecule [EP-CAM]), respectively (Fig. 4A,B). Subclusters 1–5 referred to cancer cells (Fig. 4B,D). Proliferative markers such as MKI67 were abundant in subcluster 1. Neuroepithelial markers were abundant in subclusters 1, 3, and 5 (Fig. 4B and Supplementary Fig. 2). Subcluster 5 showed the high expression of genes related to nervous system development such as NES and synaptosomal-associated protein, 25 kDa (SNAP25) (Fig. 4B). By contrast, cells in subclusters 2 and 4 showed very limited expression of the neuroepithelial markers (Fig. 4B and Supplementary Fig. 2). The Monocle 2 algorithm was employed to characterize cancer cells. The pseudotime trajectory showed that subclusters 3 and 5 exhibited similar gene expression patterns (Fig. 4C). Subclusters 3 and 5 were both enriched in ISL1 (Fig. 4B, Supplementary Table 1). Studies have shown that abnormal expression of ISL1 is closely related to the occurrence and progression of various cancers such as gastric and prostate cancers [17,18].

Furthermore, we inferred large-scale chromosomal CNVs in each single cell based on the average expression patterns across intervals of the genome. It is worth noting that the tumor cells have a higher CNV compared with other cell types (Supplementary Fig. 3, Supplementary Table 2). The CNV landscape distinguished malignant cells in subgroups/subclusters 1, 2, 3, 4, and 5 (Fig. 4D). Compared to normal cells, according to the cell chromosome fragment variation, the malignant cells were clustered into five groups, corresponding to the five subclusters (Fig. 4D). We found that cancer cells from subclusters 3 and 5 exhibited higher CNV levels than those from subclusters 2 and 4 (Fig. 4E), suggesting that ISL1-positive cells represent a more aggressive state. Therefore, we carefully investigated the enriched pathway based on ISL1-positive population makers and closely examined the proliferation and migration properties of the ISL1-positive population in the subsequent experiments.

3.4 ISL1 Modulates Neuroactive Pathway in Subclusters of Single Cell RNA-Seq Data and TCGA Patients

We performed gene ontology enrichment analyses for tumor cell subclusters. Up-regulated genes expressed in ISL1-positive population (subclusters 3 and 5) were mainly enriched for nervous system development-related pathways, such as olfactory nerve development and synaptic membrane (Fig. 5A). Up-regulated genes expressed in subcluster 1 were mainly related to cell cycle. Upregulated genes expressed in subclusters 2 and 4 were mainly enriched in ribosome functions. Furthermore, we extended the pathway analysis to TCGA UCEC RNA-seq dataset. We selected the top and bottom 10% of patients based on ISL1 mRNA abundance and performed differential gene analysis with DESeq2. This analysis identified 829 upregulated and 1648 downregulated genes between ISL1-low and ISL1-high groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified 'Neuroactive ligand-receptor interaction' as the top enriched term (Fig. 5B-D, Supplementary Table 3). In contrast, Mo-



Fig. 3. Diversity of stromal cell subtypes and expression profile of tumor cells. (A) t-SNE plot of 1451 tumor cells and 8763 stromal cells, color-coded by their associated cluster or the assigned subtype. (B–E) t-SNE plot, color-coded for relative expression (lowest expression to highest expression, gray to red) of marker genes for the T-cell (B), myeloid (C) and B-cell (D) subtypes as indicated. (E) Expression of representative marker genes of the tumor cells. *MKI67*, Antigen Kiel 67; *MZB1*, Marginal zone B; *MS4A1*, Membrane Spanning 4-Domains A1; *NNAT*, Neuronatin; *NEUROD1*, Neurogenic differentiation 1; *NES*, nestin; *SNAP25*, Synaptosome Associated Protein 25; *NCAM1*, Neural Cell Adhesion Molecule 1; *SYP*, Synaptophysin; *CHGB*, Chromogranin B; *CHGA*, Chromogranin A; *ISL1*, ISL LIM Homeobox 1; *PAX6*, Paired Box 6.

tor proteins related terms are enriched in the downregulated genes (Fig. 5C, **Supplementary Table 3**). These results suggested that *ISL1* might regulate the expression of neuroactive-related genes.

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Fig. 4. ScRNA-seq further analysis identifies distinct populations of tumor cells. (A) tSNE plot of tumor cells and fibroblasts, colorcoded by their associated cluster (left) or the assigned cell types (right). (B) Violin plots displaying the expression profile of representative known markers recently reported. (C) Pseudo-time analysis of tumor cells inferred by Monocle2. Each point corresponds to a single cell, and each color represents a tumor subcluster as indicated. (D) Heatmap showing large-scale copy number variations (CNVs) for individual cells (rows). (E) Violin plots showing distributions of CNV scores among different cell clusters and tumor subclusters.



Fig. 5. *ISL1* modulates neuroactive pathway in subclusters of single cell RNA-seq data and The Cancer Genome Atlas (TCGA) patients. (A) The enriched gene ontology terms for marker genes in each tumor subcluster. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of upregulated genes in *ISL1*-high compared to *ISL1*-low patients. (C) KEGG analysis of downregulated genes in *ISL1*-high compared to *ISL1*-high and *ISL1*-high and *ISL1*-low patients from the TCGA The Cancer Genome Atlas Uterine Corpus Endometrial Carcinoma (UCEC) cohort.

3.5 In Vitro ISL1-Positive Cells are Dynamic and Invasive

Large-scale, multicentric, multi-omics analyses of multiple types of cancer provide evidence that SCNC of different tissue origins have similar characteristics [19]. Be-

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cause there is no mature neuroendocrine tumor cell line in the field of endometrial cancer, in order to further study the effects of *ISL1* on the function of NET cells, we used the classic neuroendocrine tumor cell line H446 [20]. H446 is an established human small cell lung cancer cell line.



Fig. 6. Flow cytometric sorting of neuroendocrine tumor cells to obtain *ISL1* expression group, and explore the differences in cell function *in vitro*. (A) Flow cytometric sorting of neuroendocrine tumor cell line H446 according to the difference in the expression of *ISL1* on the cell surface. (B) The proliferation ability of H446 cells with high expression of *ISL1* is stronger than that of low expression group, (measured by cell counting kit-8 assay, ****p < 0.0001, *t* test). (C,D) The proliferation ability of H446 cells with high expression of *ISL1* is stronger than that of low expression group (verified by EdU experiment, ***p < 0.001, *t* test). Scale bars, 200 µm. (E,F) Transwell experiments showed that the cell migration ability of the high expression *ISL1* group was stronger than that of the low expression group (***p < 0.001, *t* test). Scale bars, 100 µm. (G) Representative immunohistochemistry (IHC) images of *ISL1* in small cell lung cancer (SCLC, n = 30) clinical samples. Scale bar = 5 µm. (H,I) The relation between the expression level of *ISL1* and TNM stage, *p < 0.005, *chi-square* test. EdU, 5-Ethynyl-2'-deoxyuridine.

Small cell lung cancer is an extremely aggressive neuroendocrine tumor. Correlation analysis showed that there's a high correlation between H446 gene expression profile and the neuroendocrine endometrial single cell RNA-seq data (Supplementary Fig. 4). We sorted the H446 cells by flow cytometry according to the expression level of ISL1 on the surface of the tumor cells, and obtained two groups of cells with high and low ISL1 expression for further experiments (Fig. 6A). First, we verified the proliferation function of the two groups of cells. We concluded from the CCK-8 assay that the proliferation ability of cells with high ISL1 expression was higher than that of the low ISL1 expression group (****p < 0.0001; Fig. 6B). The results of the EdU cell proliferation assay confirmed these results (***p < 0.001; Fig. 6C,D). We assessed the migration ability of the cells, and found that the proliferation ability of cells with high ISL1 expression was significantly enhanced, and the number of migrated cells quantified by the transwell experiment was much higher than that of the low ISL1 expression group (****p* < 0.001; Fig. 6E,F).

Since small cell neuroendocrine endometrial carcinoma is very rare, we thus further collected 30 SCLC patient tumor tissues and classified into early stage (stage I, II) and advanced stage (stage III, IV) according to National Comprehensive Cancer Network (NCCN) stage. IHC analysis displayed various *ISL1* expression levels in these SCLC patient tissues (Fig. 6G). In addition, we correlated the *ISL1* expression level with the clinicopathological characteristics of SCLC patients via a chi-square test. Our results showed that the expression level of *ISL1* is correlated with SCLC stages (Fig. 6H,I). All the above experiments and analysis demonstrate that tumor cells with high *ISL1* expression have stronger malignant behavior than the low expression group, suggesting that *ISL1* may have great research value and significance in NETs.

4. Discussion

The increasing morbidity and mortality rates have drawn global experts' attention to the diagnosis and treatment of EC [3]. In particular, treatment of endometrial NEC, with its unique pathological type, poses greater challenges [21,22]. This study utilized unbiased single-cell RNA-seq analysis to construct an immune atlas of EC by examining immune cells isolated from both tumor and paratumor tissues. The data revealed the intricate composition of endometrial SCNEC and endometrial epithelial cells.

We analyzed the sequencing data of patients with endometrial NEC, and then identified tumor cell subsets to explore their functions. A total of 1835 cells from clusters 2 and 9 were re-clustered into eight different subclusters. Subgroups 1–5 were cancer cells, and subgroups 6, 7, and 8 corresponded to endothelial cells (DCN), fibroblasts (COL3A1), and epithelial cells (KRT8 and EPCAM), respectively. Neuroepithelial markers were abundant in subgroups 1, 3, and 5. Subcluster 5 showed high expression

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of genes related to nervous system development, such as NES and SNAP25. By contrast, cells in subclusters 2 and 4 showed very limited expression of neuroepithelial markers.

We inferred large-scale chromosomal CNVs in each single cell based on the average expression patterns across genomic intervals. The CNV landscape differentiated malignant cells in subpopulations 1, 2, 3, 4, and 5. Compared with normal cells, malignant cells were divided into five groups, corresponding to five subgroups, according to the variation of cell chromosome segments. We found that cancer cells from subclusters 3 and 5 exhibited higher levels of CNVs than those from subclusters 2 and 4, which brought our attention to ISL1, a transcription factor belonging to the LIM/homeodomain family [23,24]. ISL1 plays a crucial role in binding to the enhancer region of the insulin gene and regulating insulin gene expression. Additionally, ISL1 is essential for the development of pancreatic cell lineages and is involved in motor neuron generation [25,26]. Mutations in this gene have been linked to maturity-onset diabetes of the young [27]. To investigate the impact of ISL1 on NETs, we conducted experiments using the NCI-446 small cell lung cancer NET cell line. By utilizing flow cytometry, we sorted the cells based on their surface expression of ISL1. Subsequently, we performed functional experiments on the sorted cells. Our findings revealed that cells with high ISL1 expression demonstrated significantly increased proliferation and migration abilities compared to the low expression group. This observation suggests that elevated ISL1 expression leads to the enhanced proliferation and migration of NET cells.

5. Conclusions

In conclusion, our study began with clinical patients and utilized single-cell sequencing analysis to observe the increased expression of *ISL1* in NET cells. The results of this study suggest that *ISL1* protein could serve as a potential biomarker for the development and advancement of NETs [28]. Moreover, the discovery of *ISL1* offers a fresh perspective on therapeutic approaches for various types of NETs.

Abbreviations

SCENC, small cell endometrial neuroendocrine carcinoma; UCEC, Uterine corpus endometrial carcinoma; SCLC, Small cell lung cancer; CCLE, Cancer cell line encyclopedia; TCGA, The Cancer Genome Atlas; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Availability of Data and Materials

The raw data and datasets generated during and/or analysis for single-cell data are available from the GEO under GSE233447. The data utilized and/or examined in the present study can be obtained from the corresponding author upon a reasonable request.

Author Contributions

YRL and YL dedicatedly conceived and designed the study, and also provided supervision. CZ conducted most of the experiments, analyzed the data, and wrote the paper. YG analyzed the data, and wrote the paper. BC provided assistance in single-cell suspension preparation procedure. QY, YS, TW and HC provided assistance for experiments. HD helped collecting SCLC patients' clinical information and provided technical assistance in image analysis of small cell lung cancer sections. YL, XW, YRL and JW conceived the idea for the project and analyzed the results. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Clinical Research Ethics Committee of the College of Medicine, Tongji University (KS2033). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10. 31083/j.fbl2903100.

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