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Review

Pulmonary Neuroendocrine Cells and Small Cell Lung Carcinoma: Immunohistochemical Study Focusing on Mechanisms of Neuroendocrine Differentiation

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Neuroendocrine (NE) differentiation has been histochemically detected in normal and cancer tissues and cells. Immunohistochemical analyses have provided a more detailed understanding of NE biology and pathology. Pulmonary NE cells are a rare lung epithelial type, and small cell carcinoma of the lung (SCLC) is a high-grade NE tumor. Pulmonary NE and SCLC cells share common mechanisms for NE differentiation. Neural or NE cell lineage-specific transcription factors, such as achaete-scute homologue 1 (Ascl1) and insulinoma-associated protein 1 (INSM1), are crucial for the development of pulmonary NE cells, and NE differentiation is influenced by the balance between Ascl1 and the suppressive neural transcription factor, hairy-enhancer of split 1, a representative target molecule of the Notch signaling pathway.

In this review, we discuss the importance of Ascl1 and INSM1 in identifying pulmonary NE and SCLC cells and introduce Ascl1-related molecules detected by comparative RNA-sequence analyses. The molecular classification of SCLC based on the expression of lineage-specific transcription or co-transcription factors, including ASCL1, NEUROD1, POU2F3, and YAP1, was recently proposed. We attempted to characterize these 4 SCLC subtypes using integrated immunohistochemical studies, which will provide insights into the molecular characteristics of these subtypes and clarify the inter- and intratumor heterogeneities of SCLC.

Key words: pulmonary neuroendocrine cells, Ascl1, small cell lung carcinoma, molecular classification, immunohistochemistry

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I. Introduction

The lung is an organ with the primary role of facilitating gas exchange between inspired air and circulating blood, and other functions include air conduction, the clearance of inhaled foreign materials, hormonal regulation, and the metabolism of xenobiotics. A well-organized, but complex, epithelial system is constructed in the airways and

alveoli to maintain these functions. At least eight principal types of airway epithelial cells have been identified in mammals: ciliated cells, basal cells, neuroendocrine (NE) cells, mucous goblet cells, club cells, serous cells, small mucous granule cells, and brush (tuft) cells [27]. Pulmonary NE cells (PNEC) are a rare and distinct cell type that is located throughout the airways and alveolar duct epithelium. Historically, PNEC were detected as clear cells (Helle Zellen) and considered to belong to a widespread endocrine system [17]. These cells were subsequently found to have an argyrophilic cytoplasm [18, 19]. The development of advances in ultrastructural research made a significant contribution to our understanding of PNEC. Bensch *et al.* [6] reported the presence of neurosecretory granules in human PNEC. Lauweryns *et al.* [40–42] investigated the PNEC of animals, described the innervation of clustered PNEC, and called the innervated structure "neuroepithelial bodies". The unified classification of "Feyrter cells" was subsequently developed, and the amine precursor uptake and decarboxylation (APUD) concept was proposed based on the common ultrastructural features of these cells and their ability to take up the active peptides present in neurons [49, 50]. The APUD family was suggested to include cells of the anterior pituitary gland and hypothalamus, pinealocytes, carotid body chief cells, thyroid C cells, lung and gastrointestinal NE cells, pancreatic islet cells, Merkel cells, melanocytes, endocrine cells of the placenta and thymus, and sympathetic ganglia cells, such as cells of the adrenal medulla. Since some of these cells lack APUD properties and the neural crest hypothesis of the APUD family was not always true, APUD was replaced with the term "diffuse NE system" [51]. With advances in immunohistochemical methods, more NE markers have been proposed, and a large number appear to be related to organelles that are specifically involved in NE differentiation and physiological functions, including cell membrane-specific proteins, dense core granule-related proteins, cytoplasmic enzymes, amine and peptide hormones, and cytoskeletal proteins [12, 15, 26, 30].

II. Immunohistochemical Features of Small Cell Lung Carcinoma

After the establishment of the APUD concept, the diffuse NE system concept in normal organs and tissues, was extended to neoplasms in the diffuse NE system. The NE differentiation of lung neoplasms was initially investigated using ultrastructural and biochemical techniques. One of the ultrastructural features of NE differentiation is dense core granules, which are synaptic vesicle-like organelles for the transportation of secretory products. The NE differentiation of pulmonary carcinoids and small cell lung cancer (SCLC) was examined ultrastructurally based on the presence of dense core granules [7, 8, 25, 28]. Pulmonary NE tumors are a distinct family of lung cancers with shared morphological, ultrastructural, immunohistochemical, and molecular characteristics, and include various histological subtypes, such as classical and atypical carcinoids, large cell NE carcinoma, and SCLC [11]. SCLC is the most malignant type among this lung cancer family, and is characterized by rapid growth and metastasis as well as NE differentiation [11, 13, 52]. Immunohistochemistry is a very useful method for accurately diagnosing small cell carcinoma, and is performed to identify NE differentiation in tumors. Chromogranin A (CHGA), synaptophysin (SYP), and neural cell adhesion molecule (NCAM) have been used as reliable NE molecules for the diagnosis of small cell carcinoma [11, 59, 60]. We identified insulinoma-associated protein 1 (INSM1) as a crucial transcription factor for the NE differentiation of SCLC, and showed that it is an excellent immunohistochemical marker for NE differentiation in tissue samples [20]. The roles of proneural transcription factors, such as achaete-scute complex homologue 1 (Ascl1) and its regulator, the Notch signaling pathway, in the regulation of NE differentiation have been examined [5, 9, 35, 36, 45]. Ascl1 is a master regulator of NE differentiation [9] and a lineage-specific oncogene of SCLC [10, 23]. Recent studies proposed that SCLC may be subclassified into 4 molecular subtypes based on the expression of lineage-specific transcription and co-transcription factors, which include ASCL1, neurogenic differentiation factor 1 (NeuroD1), POU class 2 homeobox 3 (POU2F3), and yes-associated protein 1 (YAP1) [55], with ASCL1 being the most common subtype [4, 53, 55, 56].

The findings of immunohistochemical studies on PNEC and SCLC are discussed herein with a focus on ASCL1, a representative NE differentiation-regulating molecule.

III. Histochemical, Immunohistochemical, and Ultrastructural Features of PNEC

Prior to the development of immunohistochemistry, various histochemical methods were used to identify and characterize NE cells. Silver stains were applied to display argyrophil or argentaffin reactions. Various argyrophilic stains were formulated, including the Bodian, Grimelius silver nitrate and Pascual stains. These stains appeared to work well for gastrointestinal and pancreatic NE cells; however, difficulties were associated with obtaining reproducible data on airway NE cells. In our experience, the modified Pascual's argyrophil stain [14] has been useful for identifying NE cells in the human fetal lungs (Fig. 1A) [31].

Various immunohistochemical markers for the detection of PNEC have been examined and applied to clinical settings. Until the 21st century, most of these markers have be discovered with regard to molecules associated with neural phenotypes; including cell membrane components, such as NCAM, somatostatin receptors, O_2-sensor enzyme and G-protein oα (Fig. 1B) [33], cytoplasmic components, such as neuron-specific enolase, PGP9.5, and calcium-

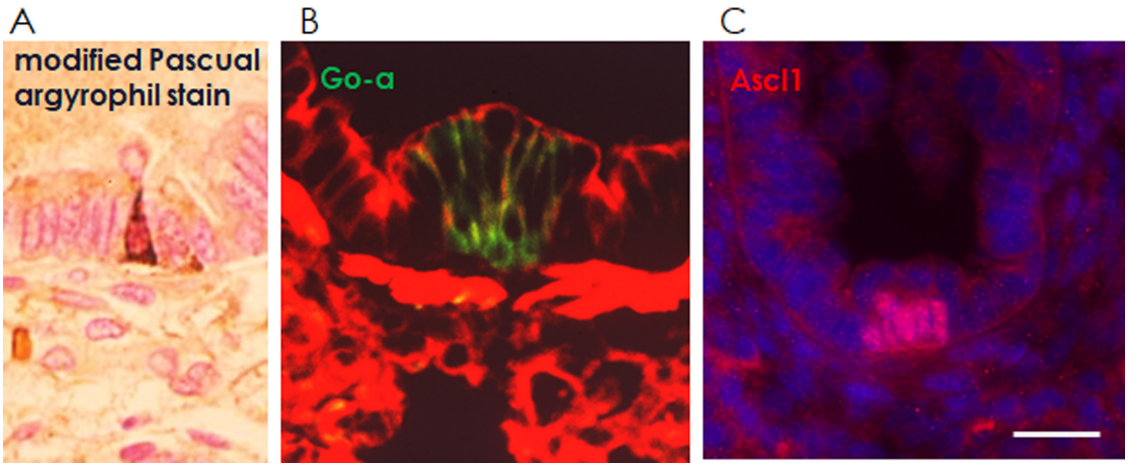

Fig. 1. Histochemical detection of pulmonary neuroendocrine cells (PNEC) in humans (**A**) and experimental animals (**B**: hamster; **C**: mouse). PNEC are distributed throughout the airway epithelium, and often form a grouped cell cluster called a neuroepithelial body. **A**: Human PNEC are detected by argyrophilic staining: the modified Pascual stain (Churukian Schenk stain). **B**: PNEC are stained with various antibodies against neuronal phenotype proteins comprising the cell membrane, secretory granule components, cytoskeletal systems, enzymes, and transcription factors. A neuroepithelial body is stained for G protein oα (Go-α; FITC). Counterstaining with rhodamine-labelled phalloidin. **C**: The nuclei of a neuroepithelial body are immunohistochemically positive for achaete-scute complex homolog 1 (Ascl1), a proneuronal basic helix-loop-helix transcription factor. TRITC, counterstained with DAPI. Bar = 20 μm.

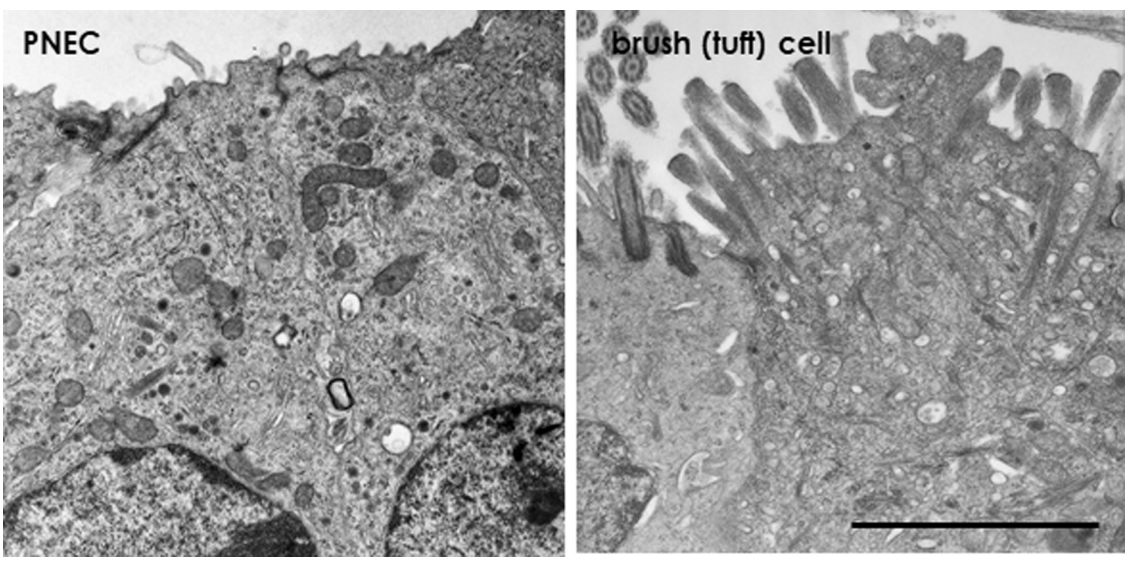

Fig. 2. Left: Ultrastructure of hamster PNEC showing well-developed organelles, including dense core granules, a rough ER, mitochondrion, and Golgi apparatus. Right: Ultrastructure of the brush (tuft) cell of a hamster bronchiole showing characteristic wide microvilli with filament bundles and developed organelles, as well as vesicular small granules. Bar = 1 μm.

binding proteins and neurofilaments, and secretory vesicle components, such as SYP, synaptobrevin, CHGA, calcitonin gene-related peptide, serotonin and gastrin-releasing peptide [12, 15, 26, 30]. On the other hand, nuclear markers for NE transcription factors, such as Ascl1 and INSM1, have more recently been used [9, 15, 34].

An ultrastructural analysis is a powerful tool for identifying PNEC because the detection of neurosecretory (dense core) granules is direct evidence of the cell type. Since brush (tuft) cells may be the origin of SCLC with low NE differentiation [29], the ultrastructures of PNEC and brush cells are presented for comparison in Figure 2 [32]. The morphological and physiological characterization of brush cells has not yet been performed in detail [54], and differences and similarities in the morphologies of PNEC and brush cells remain unclear; however, both may be the origin of SCLC. The size of the microvilli of these cells markedly differ, and some brush cells have sometimes small vesicular granules as PNECs though contents of the granules are unknown (Fig. 2).

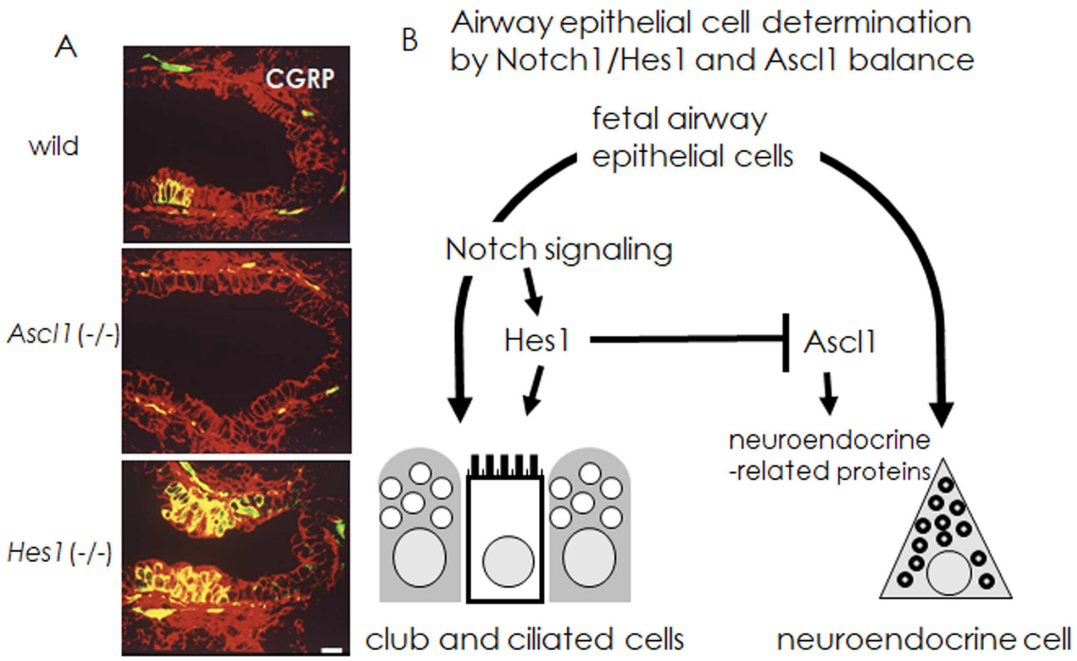

Fig. 3. **A**: Calcitonin gene-related peptide (CGRP)-positive PNEC are absent in ASCL1 gene-deficient mice (*Ascl1*(−/−)), but are abundant in Hes1 gene-deficient mice (*Hes1*(−/−)). Bar = 20 μm. **B**: A scheme showing the cell fate determination of mouse fetal airway epithelial cells by the Notch1/Hes1 and Ascl1 balance. When ASCL1 is expressed, fetal airway epithelial cells are more likely to differentiate into neuroendocrine cells. When the Notch pathway is activated and Hes1 is present, fetal airway epithelial cells do not differentiate into neuroendocrine cells and become other cell types, such as club and ciliated cells.

IV. NE Differentiation Regulated by the Balance between Ascl1 and Hairy-enhancer of Split 1 (Hes1) in the Lung

The molecular mechanisms underlying the NE differentiation of lung epithelial cells have not yet been elucidated in detail. Nevertheless, the balance between basic helix-loop-helix (bHLH) transcription factors, such as Ascl1 and Hes1, is important for predicting the PNEC lineage fate in the lungs [9, 34]. The activation of Ascl1 is crucial for NE differentiation based on the finding showing that mice deficient for the Ascl1 gene lacked PNEC [9, 34]. In contrast, PNEC differentiation was promoted in Hes1 gene-deficient mice [34]. These findings indicate that Ascl1 induces PNEC in concert with Notch signaling pathways, including Hes1, thereby influencing the PNEC cell lineage (Fig. 3). However, the balance of these two bHLH factors alone does not affect NE differentiation in the lung epithelial system because the regulatory mechanisms involved in the differentiation and proliferation of PNEC are complex and include interactions between a number of intrinsic and extrinsic factors.

V. Significance of ASCL1 in the NE Differentiation of Lung Neoplasms

The significance of ASCL1 in the NE differentiation of the mouse fetal lung epithelium and human SCLC was initially reported by Borges *et al.* [9]. A mouse experimental study showed that the overexpression of Ascl1 in lung epithelial cells induced lung neoplasms with NE features [44]. ASCL1 gene transfection has been reported in human lung adenocarcinoma cell lines, and NE differentiation was detected in ASCL1 gene-transfected adenocarcinoma cells [20, 39, 45, 48]. After the transfection of the ASCL1 gene, SYP, CHGB, and secretogranin 2 were expressed by A549 adenocarcinoma cells [39, 48], CHGA, SYP, and NCAM by H1975 and H358 adenocarcinoma cells [20], and NCAM by PC9 adenocarcinoma cells [45]. In addition to NE differentiation, ASCL1 has been suggested to play various roles in cell growth, survival, migration, tumor initiation, and epithelial-mesenchymal transition [16, 36, 38, 39, 43, 47, 48, 57, 58]. ASCL1 is a lineage-specific oncogene of SCLC [10, 23]. SCLC has recently been subdivided into 4 main subtypes based on the expression of lineage-specific transcription and co-transcription factors [55], and SCLC with the dominant expression of ASCL1 is the most common subtype [4, 53, 55, 56]. Transcriptional targets of ASCL1 were discovered using chromatin immunoprecipitation sequencing (ChIP-seq) [3, 10], which revealed many target molecules of ASCL1. These target molecules include genes related to NE differentiation, cell survival [3], and tumorigenesis [10], which suggests that ASCL1 is a lineage-specific oncogene of SCLC. A ChIP-seq analysis of ASCL1 confirmed neural and NE differentiation-related target molecules, such as gastrin-releasing peptide, INSM1,

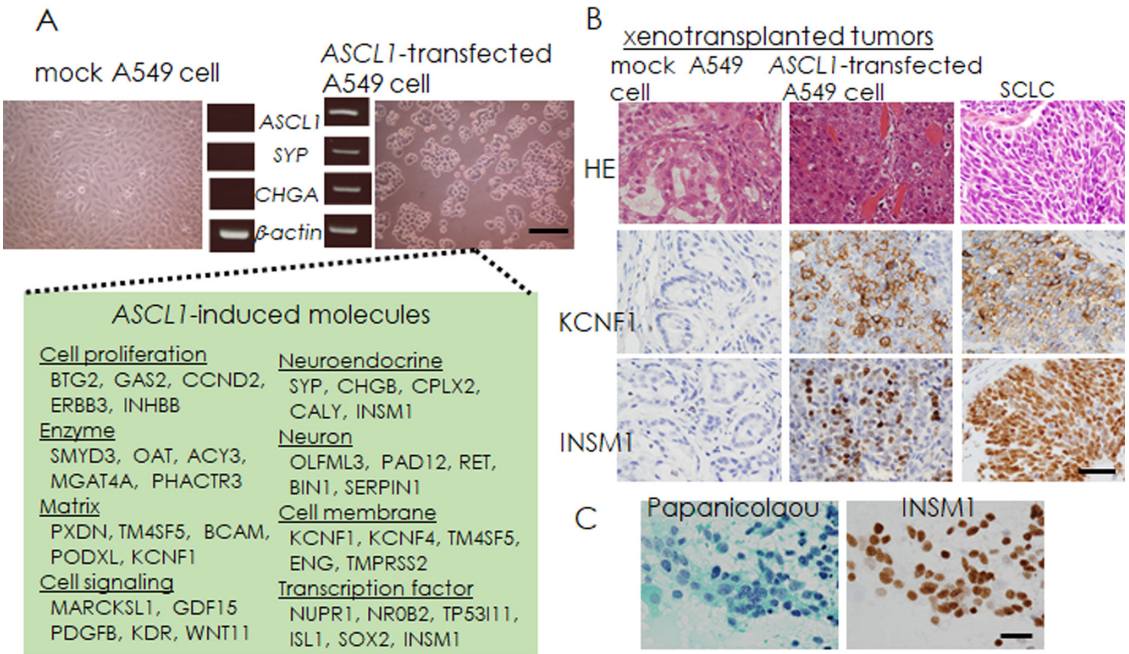

Fig. 4. **A**: Ascl1-induced neuroendocrine differentiation is established by the transfection of the ASCL1 gene into an adenocarcinoma cell line (A549). Ascl1-induced pulmonary neuroendocrine tumors show neuroendocrine differentiation, as shown in RT-RCR for synaptophysin (SYP) and chromogranin A (CHGA). In phase-contrast photographs, *ASCL1*-transfected A549 adenocarcinoma cells show changes from an adhesive to floating morphology. Bar = 50 μm. RNA sequence analyses revealed many genes related to cell proliferation, signaling, enzymes, the extracellular matrix, cell membrane, and transcription factors as well as neural or neuroendocrine differentiation by Ascl1 transfection. **B**: For example, Potassium voltage-gated channel modifier subfamily F member 1 (KCNF1) and insulinoma-associated protein 1 (INSM1), ASCL1-related molecules, are applied to immunohistochemistry on tumor tissues from mock A549 adenocarcinoma cells and ASCL1gene-transfected A549 cells (Ascl1-induced pulmonary neuroendocrine tumor) grown in immunocompromised mice, as well as surgically resected small cell lung carcinoma (SCLC) samples. Immunohistochemically, KCNF1 mainly localized to the cell membrane of ASCL1-induced pulmonary neuroendocrine tumors and SCLC samples, and INSM1 to their nuclei. Bar = 100 μm. **C**: INSM1 immunostaining is available for cytological samples. Bar = 100 μm.

and potassium voltage-gated channel subfamily members [3, 10]. Zhang *et al.* [61] examined SCLC tumors and cell lines using a 50-gene expression-based NE score, developed based on expression array and RNA-seq data. SCLC cell lines may be subdivided into two groups: classical and variant. Classical SCLC cell lines are characterized by floating cell growth in medium and high NE differentiation, and variant SCLC cell lines by adhesive growth and low NE differentiation [22, 61]. Notch activity and the loss of ASCL1 expression have been reported in variant SCLC with low NE differentiation [36, 61].

To clarify ASCL1-associated molecules, we generated ASCL1 gene-transfected A549 adenocarcinoma cell lines, which exhibited various biological phenotypes, in addition to the induction of NE differentiation [36, 39, 58]. These cells lost the adhesive growth pattern and grew with a floating growth pattern (Fig. 4A). They showed EMT phenotypes with changes in the morphology and expression of EMT-related molecules [36]. Furthermore, when xenotransplanted into skin on the backs of immunocompromised mice, these cells lost the glandular structure to gain an undifferentiated morphology (Fig. 4B). An RNA sequencing analysis of ASCL1 gene-transfected adenocarcinoma cell lines revealed a large number of ASCL1-induced molecules related to cell proliferation, enzymes, the extracellular matrix, cell signaling, neuronal and NE differentiation, the cell membrane, and transcription factors (Fig. 4A), some of which are useful for detecting ASCL1-induced NE neoplasms and small cell carcinoma (Fig. 4B). INSM1 has been employed to confirm NE differentiation in tissue and cytological samples (Fig. 4B and C) [1, 20]. In addition to a comparative RNA sequencing analysis of the mock adenocarcinoma cell line A549 and ASCL1 gene-transfected A549 (ASCL1-induced pulmonary NE carcinoma), we obtained another set of RNA-sequencing data from the H69 SCLC cell line, a classical and ASCL1-positive cell line, and the H69AR SCLC cell line, a variant, ASCL1-negative, chemoresistant H69 cell line [46]. The findings of RNA-sequencing analyses of the 2 sets of cell lines, comprising ASCL1-positive and -negative cell lines, revealed 120 common ASCL1-associated genes. These genes were analyzed by quantitated PCR analyses with 2 ASCL1-positive and 3 ASCL1-negative SCLC cell lines, 2 ASCL1 gene-transfected A549 cell lines, and 2 mock-A549 cell lines. We found 7 ASCL1-related molecules when the molecules were up-regulated in the 2 ASCL1-positive SCLC cell lines

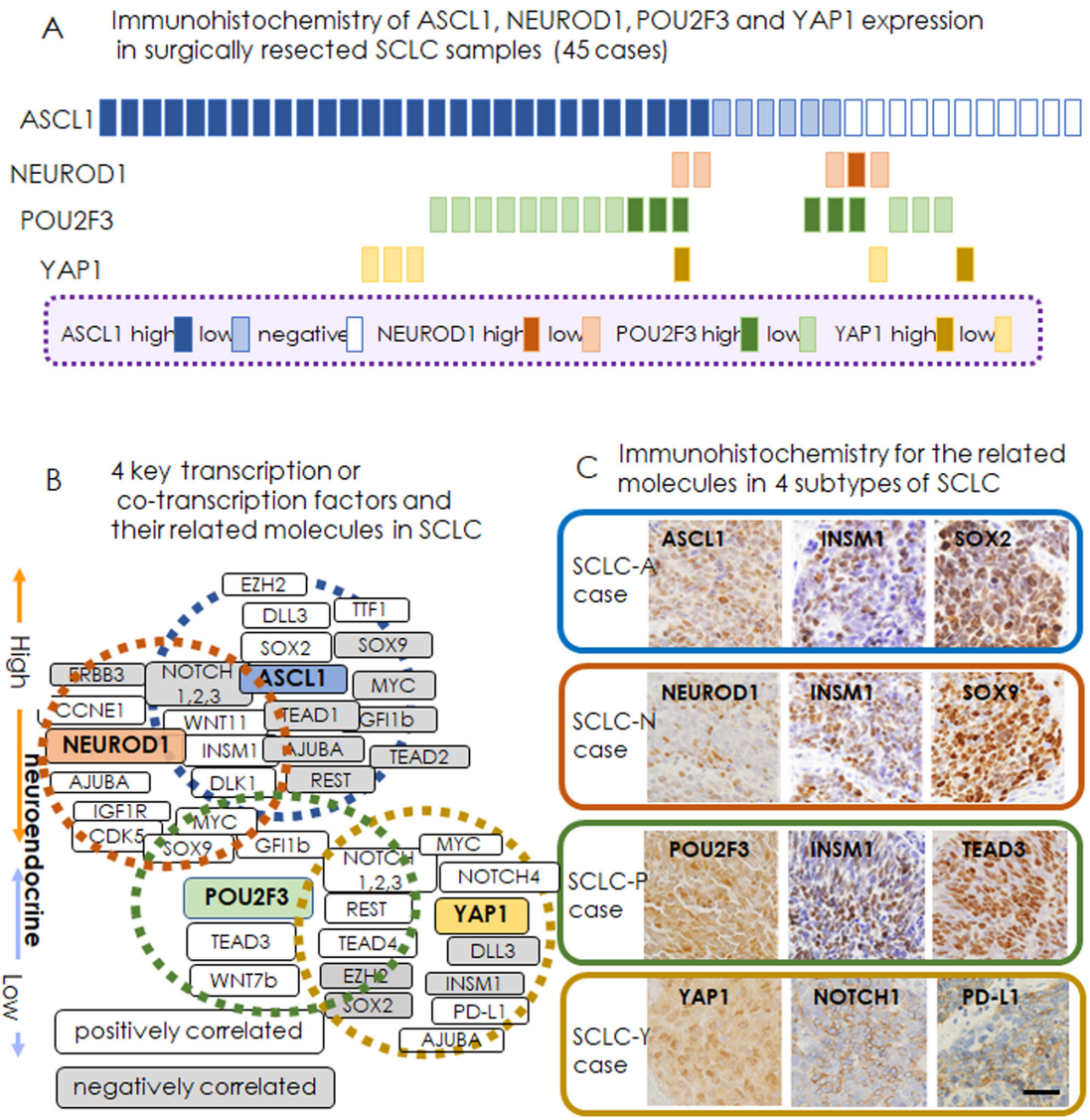

Fig. 5. The majority of SCLC express one of 4 lineage-specific transcription or co-transcription factors, including ASCL1, neurogenic differentiation factor 1 (NeuroD1), POU class 2 homeobox 3 (POU2F3), and yes-associated protein 1 (YAP1). The molecular classification for SCLC is proposed based on their expression: ASCL1 (SCLC-A), NeuroD1 (SCLC-N), POU2F3 (SCLC-P), and YAP1 (SCLC-Y) [55]. **A**: Forty-five surgically resected SCLC samples were analyzed by immunohistochemistry for the 4 key factors. The SCLC-A subtype is the most common type, and various combinations of the expression of these factors are shown. **B**: Using the transcriptome data of Asian SCLC tissue samples from the GSE60052 (n = 79) RNA sequence dataset [37]. we examined the relationships between the 4 factors and some transcription factors, such as MYC and INSM1, and signal molecules, including Notch and Wnt. Characteristic positive or negative co-relation of expression of them is detected in each subtypes of SCLC. **C**: The identification of molecules related to the 4 lineage-specific transcription or co-transcription factors was performed by immunohistochemistry. In principle, immunohistochemical results support the results of the comprehensive transcriptome study; however, SCLC-P and SCLC-Y cases do not always show low neuroendocrine subtypes (see INSM1 expression in the SCLC-P case). Bar = 100 μm.

and the 2 *ASCL1*-tansfected A549 cell lines but down-regulated in the 3 ASCL1-negative SCLC cell lines and the 2 mock A549 cell lines. These ASCL1-related molecules included INSM1, Islet1, synaptotagmin 4, Potassium channel tetramerization domain-containing protein 16, Seizure-related gene 6, Membrane-spanning 4-domain family, subfamily A8, and cordon-bleu WH2 repeat protein, and their involvement in the pathobiology of PNEC will be examined in the future [39].

VI. Immunohistochemical Analysis of Molecular Subtypes of SCLC

SCLC is a high-grade NE cancer that is characterized by rapid growth, early metastasis, high sensitivity to radiochemotherapy, the easy acquisition of chemoresistance after chemotherapy, and mutations in the TP53 and RB1 genes [2, 23, 24]. The development of more effective molecular targeted therapies is expected. Rudin *et al.* [55]

proposed molecular subtypes of SCLC based on the expression of four transcription or co-transcription factors: ASCL1, NEUROD1, POU2F3, and YAP1. According to Rudin *et al.*, ASCL1-positive SCLC (SCLC-A) and NEUROD1-positive SCLC (SCLC-N) subtypes belong to the high NE differentiation group, and POU2F3-positive SCLC (SCLC-P) and YAP1-positive SCLC (SCLC-Y) to the low NE differentiation group. However, the expression of these molecules in SCLC is not mutually exclusive, and a few SCLC cases have only one molecule. In our immunohistochemical analysis using surgically resected samples [56], more than 50% of SCLC-A cases showed positive immunostaining for NEUROD1, POU2F3, or YAP1, while the other subtypes of SCLC rarely showed a single molecule (Fig. 5A). This combined expression of the four molecules has been supported by recent findings [4, 53]. Using the transcriptome data of Asian SCLC tissue samples from the GSE60052 (n = 79) RNA sequence dataset [37], we examined the relationships between the four molecules and some transcription and signal molecules. A summary of the correlation diagram of these molecules is shown in Figure 5B. SCLC-A cases were more likely to express INSM1, DLL3, WNT11, SOX2, and EZH2, but not NOTCH receptors, REST, or YAP-related molecules. SCLC-N cases showed the positive expression of INSM1 and WNT11, similar to SCLC-A, but were more likely to express MYC and IGF1R. SCLC-P cases expressed NOTCH receptors, GFI1, and YAP-related molecules. In contrast to SCLC-A cases, SCLC-Y cases expressed NOTCH receptors, REST, and YAP-related molecules, but not INSM1, DLL3, SOX2, or EZH2 (Fig. 5B). Figure 5C shows the immunohistochemical findings of each molecular subtype. These findings often correspond with the public dataset analysis, but are not always accurate because positive staining for INSM1 has been reported in cases of the low-NE SCLC subtype (Fig. 5C). NE-lineage transcription factors, such as ASCL1 and INSM1, may be positively expressed in SCLC-P and SCLC-Y cases with NE differentiation. Although each subtype exhibits distinct vulnerability to therapeutic chemicals [21], the molecular subclassification of SCLC is not practically useful for treatment selection. In the near future, with the development of molecular target therapies, the immunohistochemical application of crucial molecules in tissue samples will provide valuable information for the assessment of cell biological and therapeutic issues in the diagnosis of SCLC.

VII. Declarations

Conflict of interests

The authors have no conflicts of interest to declare.

Ethical approval

All studies using human pathological samples followed the guidelines of the Ethics Committee of Kumamoto University (No. 342). All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

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