Perspective

# Notch signaling and Tp53/RB1 pathway in pulmonary neuroendocrine tumorigenesis

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*Comment on:* Meder L, König K, Ozretić L, *et al.* NOTCH, ASCL1, p53 and RB alterations define an alternative pathway driving neuroendocrine and small cell lung carcinomas. Int J Cancer 2016;138:927-38.

**Abstract:** Small cell lung cancer (SCLC) is a unique histological type of lung cancers, characterized by high grade malignant biological behavior and neuroendocrine differentiation. SCLC is subdivided into pure and combined types, and in the latter type, non-small cell lung cancer (NSCLC) features co-exist alongside with SCLC features. It has been reported that double mutations in Tp53 and RB1 are essential in small cell carcinogenesis, and that neuroendocrine differentiation is regulated by proneural transcription factors, such as Achaete-scute homolog 1 (ASCL1) and its signaling regulator; Notch signaling pathway. According to a recent article reported by Meder *et al.*, secondary SCLC is derived from NSCLC, with loss of Notch activity, accompanying with increased ASCL1 activity, and with further additional genetic changes in *Tp53* and *RB1*. They analyzed combined type SCLC cases, from the view point of the Notch-ASCL1-p53-RB axis, and it was the first to address comprehensively the molecular mechanisms of small cell carcinogenesis, regarding these four molecules. It is thus an urgent issue to clarify the molecular mechanisms of small cell carcinogenesis, progression, metastasis and acquisition of resistance to chemo-radiotherapy, for proper identification of a novel therapeutic target. But, in this perspective, we discussed the molecular mechanisms of small cell carcinogenesis, from the point of neuroendocrine differentiation in SCLC.

**Keywords:** Small cell lung cancer (SCLC); notch signaling; achaete-scute homolog 1 (ASCL1); *Tp53*; *RB1*

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## Introduction

Lung cancer has been reported to be one of the leading causes of cancer death worldwide, and especially among various types of lung cancer, small cell lung cancer (SCLC) is the most aggressive type, showing a rapid growth and metastasis, in spite of a temporary response to chemo-radiotherapy (1). Improvements in treatment of SCLC have not been remarkable in the past decades, and the standard chemotherapy regimen of cisplatin or carboplatin plus etopside, used for the first-line treatment of SCLC, has not changed over the past four decades (2). Fundamental studies on molecular mechanisms of small cell carcinogenesis have not been fully established, and significant progresses will be anticipated, in order to explore novel therapeutic development as soon as possible. In the recent years, few studies analyzing a relatively large scale, to search for essential and critical molecules in SCLC, were reported (3-5), and the importance of various pathways, such as cell cycle regulation associated with TP53 and RB1, receptor-kinase signaling, transcriptional network, Notch signaling, and guidance molecule system, were pointed out (3-5). In a recent article reported by Meder *et al.* (6), they proposed that secondary SCLC, could be derived from non-small cell lung cancer (NSCLC), through loss of Notch activity, accompanied with

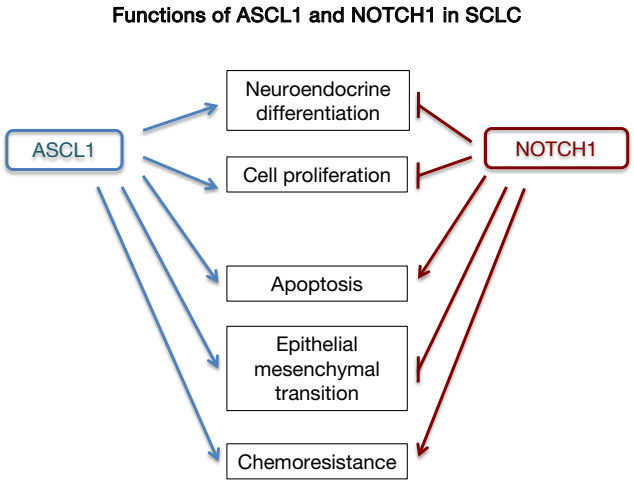


**Figure 1** Achaete-scute homolog-1 (ASCL1, termed Mash1 in rodents) is a proneural basic helix-loop-helix transcription factor, and functions in small cell lung cancer (SCLC) as not only induction of neuroendocrine differentiation, but also as a regulator of various biological activities such as cell proliferation, survival, shape, motility and chemoresistance. In the contrary, Notch signaling is involved in small cell carcinogenesis (5,6) and functions in small cell carcinoma not only to suppress neuroendocrine differentiation, but also to control various biological activities such as cell proliferation, survival, shape, motility and chemoresistance.

increased achaete-scute homolog 1 (ASCL1) activity, with further additional genetic changes in *Tp53* and *RB1*. They analyzed combined type SCLC from the view point of the Notch-ASCL1-p53-RB axis, and—to our knowledge—it was the first article to discuss comprehensively the molecular mechanisms of small cell carcinogenesis regarding these four molecules. This article is very interesting as it reports (I) SCLC could be generalized basically by changes in the Notch-ASCL1-p53-RB axis; (II) pre-acquisition of potential neuroendocrine differentiation through modulating Notch-ASCL1 balance seems to be important in the development of SCLC; and (III) there could be an alternative pathway in small cell carcinogenesis.

In the current perspective, although many issues remain to be solved for understanding the molecular mechanisms of carcinogenesis of SCLC, and making reference to the article reported by Meder *et al.* (6), we will discuss some of the recent insights into the mechanisms of neuroendocrine differentiation, and expand the argument on small cell carcinogenesis.

## Transcriptional regulation of neuroendocrine differentiation

Pulmonary neuroendocrine cells are specialized epithelial cells, distributed sparsely throughout the lung epithelia, from the bronchus to the bronchio-alveolar junctional area, and could serve to maintain the homeostasis of airway microenvironments (7,8). Various transcription factors have been reported to determine neuroendocrine differentiation in the normal and neoplastic lung epithelial cells, and ASCL1; a proneural basic helix-loop-helix transcription factor, has been regarded as a neuroendocrine inducer and lineage marker (9-12). In normal epithelial cells, transfection of *ASCL1* gene directed epithelial cell toward neuroendocrine differentiation, and in a lung adenocarcinoma cell line, cell morphology and proliferation activity were altered by *ASCL1* transfection (6,13,14). ASCL1 appears to be involved in the cell growth, survival, differentiation, cell adhesion, and chemoresistance (*Figure 1*). According to Osada *et al.* (14), inhibition of *ASCL1* suppressed cell proliferation and induced apoptosis in SCLC cell lines, which could signify that ASCL1 plays pivotal role in carcinogenesis of SCLC. Expression of ASCL1 is suppressed by Notch signaling in normal epithelial cells and cancer cells (15-17), and Hes1; one of the representative target genes of Notch signaling pathway and a repressive basic helix-loop-helix transcription factor, is a strong suppressor of ASCL1 in developing mouse lung and in SCLC cells (10,18). One of the ASCL1 candidate regulators in SCLC cells is Repressor element-1 silencing transcription factor (REST, as it suppresses the expression of ASCL1 through epigenetics mechanisms in neurogenesis (19). Besides, REST is deficient in SCLC cell lines (20).

In addition to ASCL1, Brain 2 (BRN2); a POU domain transcription factor, is a developmentally neural-cell specific factor, and could participate in neural differentiation of SCLC cells (18,21). Recently, a zinc-finger transcription factor; insulinoma-associated protein 1 (INSM1), was reported as a crucial regulator for neuroendocrine differentiation for normal lung epithelial cells (22) and SCLC cells (18), and INSM1 could regulate the expression of both ASCL1 and BRN2 in lung cancer cell lines (18). In addition, INSM1 alone can induce neuroendocrine differentiation in NSCLC cell lines (18). Moreover, the expression of INSM1 and ASCL1 was suppressed by the activation of Notch signaling (18).

Another transcription factor that regulates neuroendocrine differentiation in lung cancer cells, is retinoblastoma (RB)

gene product. There is an interesting report, which showed that increased pulmonary neuroendocrine cells are observed in *Rb1* gene-deficient mouse lungs (23). Considering that *RB1* is one of the essential genetic abnormalities in SCLC, an attractive molecular research field for studying the relation between neuroendocrine differentiation and *RB* abnormalities remains to be explored. In addition, pulmonary neuroendocrine cell hyperplasia in *Rb1* gene-deficient mice disappears with loss of of *E2f3*, one of Rb1 targets (24).

## Significance of Notch1 in small cell carcinoma

Notch signaling is one of the most important cell signaling system, and through interaction with ligands of the Delta and/or Jagged/Serrate families, it regulates several genes such as *Hes1*, *cyclinD1*, *c-Myc* and *Akt* (25). The importance of Notch signaling in carcinogenesis has been reported in controlling the differentiation, metabolism, cell cycle progression, angiogenesis, stemness, and of cancer cells (26). In lung cancer, Notch exhibits both tumor promoting and suppressive functions. A whole genome sequencing study of SCLC cases revealed mutations of Notch family genes in about 25% of the cases examined, suggesting a tumor suppressive nature of *Notch* in SCLC cells (5). In SCLC cell lines, gene transfection and knockdown experiments clarified that Notch1 plays significant role in suppression of cell proliferation, enhancement of apoptosis, induction of epithelial morphology (mesenchymal-epithelial transition), suppression of motility, acquisition of drug resistance and suppression of neuroendocrine differentiation (*Figure 1*) (17,27-29). Regarding cell fate determination, Notch1-Hes1 pathway is a repressor of neuroendocrine differentiation through decreased expression of ASCL1 and INSM1 (10,16,18,30). Using immunohistochemistry, pulmonary neuroendocrine cells are positive for Ascl1, but negative for both Notch receptors and Hes1, while lung non-neuroendocrine cells are negative for Ascl1, but positive for Notch receptors and Hes1 (*Figure 2A*). This mutually exclusive expression pattern is true in lung cancers; as also confirmed by western blotting analyses, which revealed that SCLC cell lines are positive for ASCL1 and/or INSM1, but negative for Notch1, and NSCLC cell lines are negative for ASCL1 and/or INSM1, but positive for Notch1.

The combined type SCLC has both SCLC and NSCL compartments (18). Immunohistochemically, the SCLC compartment is positive for INSM1, but negative for Notch1, and the NSCLC compartment is negative for INSM1, but positive for Notch1 (*Figure 2B*), which suggests that Notch signaling pathway is important in determination of the subtypes of SCLC. Some molecular mechanisms of down-regulation of ASCL1 by Notch signaling have been proposed. The human *ASCL1* promoter region has broad transcriptional enhancer and tissue-restricted transcriptional repressor motifs (31), and the repressor motif, an N-box sequence, is sensitive to Notch signaling activity via Hes1 binding (32). Moreover, Notch signaling can induce degradation of ASCL1 through proteasome activation (15). A combination of inactivation of Notch signaling, with expression of ASCL1, direct lung epithelial cells to a neuroendocrine phenotype (30), and this molecular relationship seems to be essential in the origin of SCLC (6).

## Molecular mechanisms of small cell carcinogenesis

Frequent mutations in both *TP53* and *RB1* have been identified in SCLC cells (3-5), and thus it seems reasonable that genetic events with the bi-allelic TP53 and *RB1* mutations must determine the process of small cell carcinogenesis. Actually, the experimental study by Meuwissen *et al*. (33) showed that mice carrying conditional alleles for both *Trp53* and *Rb1* developed small cell carcinoma in the lung, which supports the fact that inactivation of both *Tp53* and *RB1* is a prerequisite event for the pathogenesis of SCLC. Following this study, several mouse models which developed SCLC, have abnormalities in both *p53* and *Rb1* genes, and detailed analyses of the pathobiology of these SCLC in these models is useful to understand human SCLC (34). According to Meder *et al*. (6), SCLC has two oncogenic pathways: primary SCLC, which comes from neuroendocrine precursor cells, with bi-allelic TP53 and RB1 mutations, and secondary SCLC, which comes from Notch-defective NSCLC, which already has *TP53* mutations and acquire additional *RB* inactivation.

Regarding to the origin of primary SCLC, they consider that neuroendocrine precursor cells -which are characterized by inactivation of Notch signaling and ASCL1 expression- are the origin of primary SCLC. This hypothesis could be accepted, as in mouse developing lungs, ASCL1 expressing cells could be a progenitor for various epithelial and mesenchymal cells (11), and could have migrating activity (12). However, using cell lineage-restricted Adeno-Cre virus, Sutherland *et al*. (35) showed clearly that loss of *Tp53* and *Rb1* could efficiently transform

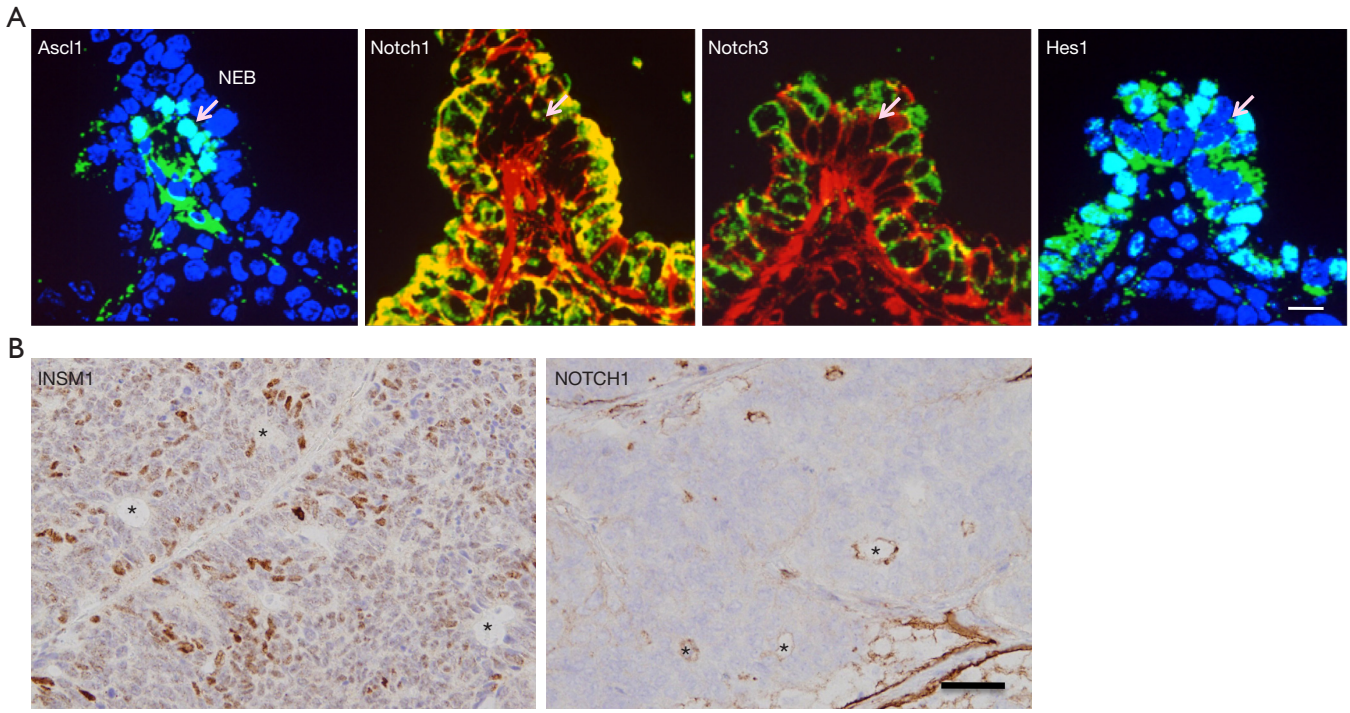


**Figure 2** Notch and neuroendocrine transcription factors in fetal mouse lung (A) and human combined type of small cell lung cancer (SCLC) (B). (A) Serial sections of fetal mouse lung tissues immunostained for Ascl1, Notch1, Notch3, and Hes1. A cluster of pulmonary neuroendocrine cells (arrow; termed neuroepithelial body, NEB) are positive for Ascl1, but negative for Notch1, Notch3 and Hes1 in the nuclei, but non-neuroendocrine cells are negative for Ascl1, but positive for Notch1, Notch3 and Hes1. Counterstained with DAPI in the sections for Ascl1 and Hes1, and stained with rhodamine-labelled phalloidin in the sections for Notch1 and Notch3. Bar =20 μm; (B) combined type of SCLC immunostained for INSM1 and NOTCH1. The small cell carcinoma component is stained for INSM1, but not for NOTCH1. On the other hand, the adenocarcinoma component forming lumens (asterisks) are negative for INSM1, but positive for NOTCH1. Counterstained with hematoxylin. Bar =20 μm. SCLC, small cell lung cancer.

neuroendocrine, Clara and type 2 alveolar cells into SCLC cells (35). This study suggests that these epithelial cell lineages could be the origin of SCLC, and that inactivation of Notch signaling and ASCL1 expression are not always necessary to initiate SCLC development. Regarding to the origin of secondary SCLC, transformation from NSCLC to SCLC has been noticed, as a result of acquisition of resistance mechanisms against EGFR tyrosine kinase inhibitors (36). Niederst *et al.* (37) reported that such transformation from adenocarcinoma to SCLC always accompany the loss of *RB1* gene, yet *RB1* gene knockdown did not induce neuroendocrine differentiation in the EGFR mutant adenocarcinoma cell line. The combined SCLC cases presented by Meder *et al.* (6), suggest that NSCLC harboring Notch abnormalities, could become SCLC, with the addition of *RB1* gene mutations, although abnormalities were reported in Notch2 but not in Notch1, and that inactivation of Notch2 is not a strong inducer of neuroendocrine differentiation (16). Considering that adenocarcinoma with mutant EGFR could transform to SCLC with the addition of *RB1* gene abnormalities, the second and alternative pathway could be important in carcinogenesis of combined type SCLC. However, it is necessary to emphasize that combined type SCLC could originate from pure SCLC, and in this context, NSCL component should have active Notch signaling pathway and decreased ASCL1/INSM1 expression, contrary to Notch signaling inactivation and ASCL1/INSM1 expression seen in SCLC (18). Meder *et al.* (6) seems to emphasize -in their article- that a combination of inactivation of Notch signaling, with Ascl1 expression, precedes *RB1* gene mutation, in small cell carcinogenesis,

 

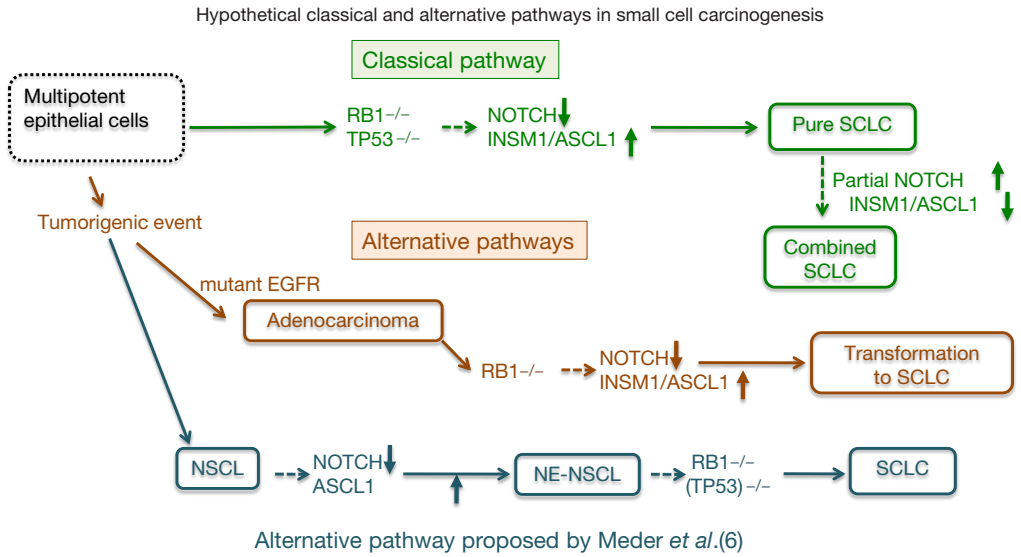


**Figure 3** Hypothetical classical and alternative pathways in small cell carcinogenesis. In the classical pathway, small cell lung cancer (SCLC) could arise from lung epithelial cells with abnormalities in both *TP53* and *RB1*, followed by inactivation of Notch signaling and expression of Achaete-scute homolog 1 (ASCL1)/INSM1. When reactivation of Notch signaling occurs, non-small cell lung cancer (NSCLC) could appear to make the combined type. On the contrary, in the alternative pathways, SCLC could arise from pre-exisitent NSCL. Transformation of SCLC from adenocarcinomas with mutant EGFR after molecular-targeted treatments is well documented, and RB1 gene deletion seems to be necessary to make SCLC. In another alternative pathway, inactivation of Notch signaling with ASCL1 expression precede RB1 abnormalies, to produce SCLC from NSCLC (6).

in both the classical and the alternative pathways. This issue may be similar to the question of which came first; the chicken or the egg. As SCLC could arise from different cell lineages, other than neuroendocrine precursor cells (35), the premise of inactivation of Notch signaling and ASCL1 expression is not always necessary to be considered. Prerequisite of genetic alterations in Tp53 and RB1 should be also crucial in the classical pathway for small cell carcinogenesis, and could be important in the alternative pathway in SCLC transformation from adenocarcinoma with mutant EGFR (*Figure 3*). It is an attractive research filed to clarify molecular network linking the inactivation of Notch signaling pathway, with ASCL1/INSM1 expression and *RB1* gene abnormalities.

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