NOTCH, ASCL1, p53 and RB alterations define an alternative pathway driving neuroendocrine and small cell lung carcinomas

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Small cell lung cancers (SCLCs) and extrapulmonary small cell cancers (SCCs) are very aggressive tumors arising de novo as primary small cell cancer with characteristic genetic lesions in RB1 and TP53. Based on murine models, neuroendocrine stem cells of the terminal bronchioli have been postulated as the cellular origin of primary SCLC. However, both in lung and many other organs, combined small cell/non-small cell tumors and secondary transitions from non-small cell carcinomas upon cancer therapy to neuroendocrine and small cell tumors occur. We define features of “small cell-ness” based on neuroendocrine markers, characteristic RB1 and TP53 mutations and small cell morphology. Furthermore, here we identify a pathway driving the pathogenesis of secondary SCLC involving inactivating NOTCH mutations, activation of the NOTCH target ASCL1 and canonical WNT-signaling in the context of mutual bi-allelic RB1 and TP53 lesions. Additionally, we explored ASCL1 dependent RB inactivation by phosphorylation, which is reversible by CDK5 inhibition. We experimentally verify the NOTCH-ASCL1-RB-p53 signaling axis in vitro and validate its activation by genetic alterations in vivo. We analyzed clinical tumor samples including SCLC, SCC and pulmonary large cell neuroendocrine carcinomas and adenocarcinomas using amplicon-based Next Generation Sequencing, immunohistochemistry and fluorescence in situ hybridization. In conclusion, we identified a novel pathway underlying rare secondary SCLC which may drive small cell carcinomas in organs other than lung, as well.

Key words: Lung cancer, small cell lung cancer, achaete-scute homolog 1, neurogenic locus notch homolog, retinoblastoma protein

Abbreviations: AdC: (pulmonary) adenocarcinoma; ASCL1: Achaete-scute homolog 1; CDK: cyclin dependent kinase; DKK1: Dickkopf 1; eV: empty vector; FISH: fluorescence in situ hybridization; FFPE: formalin-fixed paraffin-embedded; IF: Immunofluorescence; IHC: immunohistochemistry; LCNEC: (pulmonary) large cell neuroendocrine carcinoma; LRP6: low density lipoprotein receptor-related protein-6; NE: neuroendocrine; NSCLC: non-small cell lung cancer; p53: tumor protein 53; RB: retinoblastoma protein; SCC: small cell cancer; SCLC: small cell lung cancer; SqCC: (pulmonary) squamous cell carcinoma; WNT: wingless-type

Additional Supporting Information may be found in the online version of this article.

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The current WHO classification of lung cancer discriminates small cell lung cancer (SCLC) from non-small cell lung cancer (NSCLC) comprising the entities adenocarcinoma (AdC), squamous cell carcinoma (SqCC), a few rare subtypes of NSCLC, large cell neuroendocrine carcinoma (LCNEC), and finally typical and atypical carcinoids. A novel genomics-based taxonomy of lung tumors proposed by the worldwide initiative of the Clinical Lung Cancer Genome Project (CLCGP) and the Network Genomic Medicine (NGM) suggests that a combination of histological and genomic denominators will redefine the classification into SCLC/LCNEC, AdC, SqCC and carcinoids.

SCLC has distinct pathological and clinical features. Tumor cells have round, spindled nuclei with finely granulated chromatia, inconspicuous nucleoli, scant cytoplasm, and frequently shows nuclear moulding. SCLCs have high mitotic rates (>60 mitoses per 2 mm²) and frequently a neuroendocrine (NE) phenotype. All small cell carcinomas (SCCs), however representing a rare tumor entity, share a very aggressive biology with early systemic spread, irrespective of organ of origin. Therefore, it is likely that general molecular mechanisms drive “small cell-ness” with cancer stem cell-related features.

We and others showed that mutual bi-allelic TP53 and RB1 alterations are central events in SCLC biology. Bi-allelic loss of TP53 and RB1 is sufficient to induce a SCC phenotype in murine lung tumors. Nevertheless, combined lung carcinoma phenotypes and relapses with a changed phenotype upon cancer therapy occur in patients. Thus, we suggest that NE SCCs may not only arise as primary lesions or as a synchronous combined carcinoma but also arise as secondary lesions in form of relapses originating from non-small cell carcinomas induced by cancer therapy.

Achaete-scute homolog 1 (ASCL1) is a basic-helix-loop-helix transcription factor pivotal for NE differentiation and expressed in pulmonary NE cells and in SCLC. Moreover, ASCL1 promotes more aggressive AdC growth in vivo and may interact with the central "retinoblastoma protein-tumor protein 53" (RB-p53) axis in the carcinogenesis of NE lung cancers. ASCL1 contributes to enhanced proliferation and migration in lung cancer cells in vitro by targeting cyclin-dependent kinase 5 (CDK5).

ASCL1 expression is regulated downstream of neurogenic locus notch homolog (NOTCH) signaling mediated through four different receptors which causes polyubiquitination-mediated ASCL1 degradation. Altered NOTCH-signaling by receptor mutations is frequently found in cancer. Thereby the mutated domain determines the functionality, for example, activating mutations located in the Proline Glutamic acid Serine Threonine rich (PEST) domain or inactivating mutations in the EGF-like and ankyrin (ANK) repeats.

We defined features of “small cell-ness” and investigated signaling via the NOTCH- and ASCL1-dependent pathway in vitro. We then performed amplicon-based Next Generation Sequencing (NGS) of different tumor sample cohorts to identify NOTCH mutations. Large parts of the genomic Guanine Cytosine (GC) rich NOTCH1-4 loci are difficult to sequence and hence, data from whole genome sequencing and The Cancer Genome Atlas (TCGA) are not fully informative.

Taken together, our data suggest that there are two oncogenic pathways for NE SCCs. Primary SCLC originates from NE stem cells with mutual bi-allelic TP53 and RB1 alteration in contrast to secondary SCLC developing from NOTCH-defective NSCLC that already harbor TP53 mutations and acquire additional RB inactivation.
Immunofluorescence (IF) and Immunohistochemistry (IHC)
For IF cells were plated on cover slips, fixed in 4% PBS-buffered formalin and pre-treated with 0.25% TritonX. Staining was performed for 30 min in a humidified chamber at RT. Images were taken by an inverted microscope fitted with an ApoTome (Zeiss). IHC stain was performed as previously described.17

Fluorescence in situ hybridization (FISH)
FISH was performed as previously described18 RB1 probe (red) (artificial BAC clone: RP11-893E5, Life Technologies) and chromosome 13 centromeric probe (green) (Empire Genomics) were used. Evaluation of RB1 deletions in 100 tumor cells was performed by fluorescence microscopy using ×60 magnification (Zeiss).

AmpliCQn-based NGS of formalin-fixed paraffin-embedded tumor samples
Formalin-fixed paraffin-embedded (FFPE) tumor samples were obtained from our routine diagnostics with approval of the local ethics committee (Ref Number: 10-242). Ion AmpliSeqTM Custom DNA Panels (Life Technologies) were designed (Supporting Information Table S3) and used and analyzed according to manufacturers instructions with modifications.19

Statistics
Statistics were calculated using Excel (Microsoft), Graph Pad Prism (STATCON) and SPSS (Armonk). We used two-sided Students t test. If normal distribution and similar variance in an experiment were not applicable, Kruskal-Wallis-Test was used. Error bars indicate standard error of the mean (SEM).

Results
Establishment of features of “small cell-ness” according to lung cancer cell lines
Pathological and clinical features of SCLC were described in patients based on IHC20 and integrative genome analysis.6 We adapted criteria of markers, mutations and morphology characteristic for SCLC to establish features of “small cell-ness,” especially for in vivo studies (Fig. 1).

However, intermediate forms of lung carcinomas with large cells but NE differentiation such as LCNECs were of special interest. Finally, we categorized the two cell lines, DMS114 and SW1271, declared as SCLC, as LCNEC, since they did not completely meet the features of “small cell-ness.”

First, we analyzed expression of classical NE markers Chromogranin A, Synaptophysin and CD56 (Figs. 1a–1c). We also included ASCL1 (Fig. 1d), since reanalysis of previously published expression-array data of NSCLC and SCLC samples21,22 identified significant expression of ASCL1 in SCLC. Additionally, we determined RB protein expression (Fig. 1e).

At least one NE marker was expressed by cell lines categorized as SCLC (green) and LCNEC (blue). RB protein was expressed in AdC (black) and LCNEC cell lines but nor on SCLC cell lines.

We used amplicon-based NGS to identify mutations on RB1 and TP53 (Fig. 1f). Mutual RB1 and TP53 mutations were only identified in SCLC cell lines. Thereby RB1 mutations correlated with the lack of RB protein expression. Using different amplicon-based panels, we identified other oncogenic mutations, for example EGFR mutations in PC9 and H1975.

To determine cell morphology, we used monolayer cell culture and flow cytometry measuring cell size by forward scatter (FSC-A) (Fig. 1g).

The three analyzed AdC cell lines (A549, PC9 and H1975) grew adherently, had cells with spindle shape, abundant cytoplasm and a mean FSC-A of 151K referring to large cell size. LCNEC cell lines (H460, DMS114 and SW1271) grew adherently, had round to spindle formed or irregular shaped cells with intermediate cytoplasm. Mean FSC-A was 130K. SCLC cell lines (GLC1, GLC8 and N417) grew in cell clusters in suspension, had round cells with scant cytoplasm and a mean FSC-A of 90.2K referring to small cell size.

Taken together, the features of “small cell-ness” clearly describe the most prominent characteristics of SCLC and may represent a model to pre-categorize SCLC and LCNEC, especially for in vitro experiments. Thus, we postulate a general phenotype also of extrapulmonary small cell carcinomas (SCCs) that comprises NE marker expression including ASCL1, mutual RB1 and TP53 mutations and small round clustered cells.

ASCL1 overexpression induced a small cell carcinoma phenotype and canonical WNT-signaling
ASCL1 is a NE master regulator and expressed in multipotent stem cells of NE lineage. Lineage decisions in lung development are triggered by NOTCH-signaling upstream of ASCL1 down-regulation.23 Thus, we hypothesized that ASCL1 may be a key-factor in SCC development.

We transfected PC9 cells (AdC) with an expression plasmid containing ASCL1 or vector control (Fig. 2). PC9 cells harbor an activating EGFR mutation and an inactivating TP53 mutation and may therefore represent a model system for secondary SCLC, as clinically observed after intensive treatment AdCs relapse as SCLC.

Stable transfectants only harboring the empty vector (eV) and three clones overexpressing ASCL1 (c1-c3) were derived (Fig. 2a). We analyzed ASCL1 clones for features of “small cell-ness.” Prior to experiments we controlled clonal origin by microsatellite analysis referring to the parental PC9 cell line (Supporting Information Fig. S1).

ASCL1 clones showed at least 50-fold increased ASCL1 expression (Fig. 2b). Interestingly, Dickkopf 1 (DKK1) expression was significantly decreased upon ASCL1 expression (Fig. 2c). Moreover, cell proliferation was significantly
Figure 1. Establishment of “small cell-ness” features for lung carcinoma cell lines analyzing markers, mutations and morphology. NSCLC-AdC (black), LCNEC (blue) and SCLC (green) were compared. NE marker expressions of (a) Chromogranin A (CHGA), (b) Synaptophysin (SYN), (c) CD56 and (d) ASCL1 were determined by qRT-PCR and calculated by ΔΔCT-method. (e) RB protein expression determined by Western Blot. (f) Mutations identified by amplicon-based NGS. Allelic fraction was listed in %. (g) Cell morphologies determined in monolayer cell culture by microscopy. Bars indicate 100 μm. Cell size determined by forward scatter (FSC-A) properties measured by flow cytometry.
enhanced in ASCL1 clones compared to eV (Fig. 2d) and they expressed CD56 (Fig. 2e). Remarkably, ASCL1 overexpression directly induced a switch towards SCLC-like cell morphology (Fig. 2f).

We hypothesized that ASCL1 activates pro-proliferative WNT-signaling because ASCL1 directly represses transcription of DKK1, a negative WNT-signaling pathway regulator. DKK1 acts as a corepressor of low density lipoprotein receptor-related protein-6 (LRP6), a co-receptor recruited by Frizzled to canonically transduce WNT-signals into the cell. Thus, we analyzed WNT-signaling and the phosphorylation of LRP6 (Fig. 2g).

We found robust induction of phospho-LRP6 (pLRP6) and the WNT targets CyclinD1 and c-Myc and moderate reduction of Glykogen synthase kinase 3β (GSK3β) in ASCL1 clones compared to eV.

Effects of WNT-pathway inhibition in cancers are tested in phase I clinical trials, for example in previously treated NSCLC patients (ClinicalTrials.gov Identifier: NCT01957007, Bayer) or in patients with other malignancies (ClinicalTrials.gov Identifier: NCT01351103, Novartis).

Consistently, upon treatment with WNT-inhibitor IWP-2 the ASCL1 clone showed significantly reduced cell proliferation in a dose-dependent manner whereas the eV control...
remained unaffected with respect to cell growth (Fig. 2h). Inhibition of WNT-signaling was controlled by qRT-PCR of Cyclin D1 and Axin2 (Supporting Information Fig. S2).

Moreover, we showed induction of apoptosis by 7AAD and AnnexinV stain upon WNT-pathway inhibition in two SCLC cell lines, GLC1 and GLC2, in a dose-dependent manner (Supporting Information Fig. S3). Thus, WNT-signaling provided a potential therapeutic target in SCLC.

In conclusion, overexpression of ASCL1 was sufficient to trigger canonical WNT-signaling via phosphorylation of the coreceptor LRPs, to induce NE differentiation and to mediate the phenotypic switch towards “small cell-ness,” which did not result from an accidental de novo mutation in RB1 (data not shown).

ASCL1 triggered phosphorylation of RB by CDK5

ASCL1 clones presented a SCC phenotype and fulfilled the criteria of “small cell-ness” apart from mutual RB1 and TP53 mutation, since de novo mutations in RB1 were not acquired.

In addition to direct genetic inactivation, RB can be inactivated by phosphorylation.26 Thus, we performed Western blot analysis to determine total RB protein and phosphorylation status. All three ASCL1 clones showed higher expression of phosphorylated RB at Serine (Ser) 780, Ser795, Ser807/811 than PC9 eV cells (Fig. 3a). Total RB protein expression, however, remained unchanged. Thus, we concluded that ASCL1 overexpression caused inactivation of RB by phosphorylation. Phosphorylation of RB is triggered by CDKs. We found upregulated CDK5 in ASCL1 clones compared to eV control. In contrast, CDK2 protein levels were not altered by ASCL1 overexpression and CDK4 and CDK6 expression was reduced (Fig. 3a).

Based on these data, we further validated CDK5 as a driver of RB phosphorylation in ASCL1 clones using Roscovitine to selectively inhibit CDK5 activity (Figs. 3b–3d). CDK5 inhibition did not affect mitotic cell count and also did not induce cleaved Caspase 3 referring to apoptosis. Analysis of RB, pRBSer780, pRBSer795, pRBSer807/811, CDK5 and pCDK5Ser159 expression showed significantly decreased levels of CDK5 and pCDK5Ser159 and RB phosphorylation. To further elucidate the connection between ASCL1, CDK5 and pRB, we performed siRNA mediated knock-down of ASCL1 in a SCLC cell line (GLC8) and a LCNEC cell line (DMS114). Consistently, ASCL1 knock-down reduced protein levels of CDK5 and of pRBSer780 (Supporting Information Fig. S4).

Since ASCL1 is targeted by NOTCH-signaling, we performed siRNA mediated knock-down of NOTCH1 and NOTCH2 in PC9 cells (Supporting Information Fig. S5). We observed significantly increased ASCL1 and CD56 expression. Flow cytometry revealed stable RB protein expression and significantly increased RB phosphorylation at Ser780, but not as evident as in ASCL1 clones.

Nevertheless, NOTCH1 and NOTCH2 knock-down were not potent enough to cause a phenotypic switch towards SCC and cell proliferation was even reduced, since PC9 cells originally harbored intact NOTCH-signaling (Supporting Information Fig. S5).

To prove direct effects of RB inactivation on SCC growth, we performed siRNA mediated knock-down of RB in A549 and PC9. A549 cells (p53 wild-type) showed reduced number of mitotic cells positive for phospho-histone H3 (pH3) and increased apoptosis (cleaved Caspase 3 – cCaspase3) determined by flow cytometry upon CDK5 inhibition by Roscovitine for 48 hrs. (d) RB and CDK5 protein levels measured by flow cytometry. Mean fluorescence intensity was normalized on secondary antibody control (Index). Data are presented as mean ± SEM of analysis in triplicates. Statistical significance was calculated using the Student’s t test, two-sided, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 3. ASCL1 mediated phosphorylation of RB triggered by CDK5. (a) RB and CDK protein levels determined by Western blot. (b+c) Mitotic cell number (pHistoneH3 – pH3) and induction of apoptosis (cleaved Caspase 3 – cCaspase3) determined by flow cytometry upon CDK5 inhibition by Roscovitine for 48 hrs. (d) RB and CDK5 protein levels measured by flow cytometry. Mean fluorescence intensity was normalized on secondary antibody control (Index). Data are presented as mean ± SEM of analysis in triplicates. Statistical significance was calculated using the Student’s t test, two-sided, * p < 0.05, ** p < 0.01, *** p < 0.001.

Conclusively, ASCL1 assists the central RB-p53 signaling axis in the establishment of a SCC phenotype.
Mutational patterns of RB1, TP53 and NOTCH genetic alterations in SCLC, SCC, LCNEC and AdC

We hypothesized a central signaling axis of NOTCH inactivation, ASCL1/CDK5 activation and mutual RB/p53 inactivation. Since isolated NOTCH knock-down was not potent enough, we suggested that genetic lesions may be driver events in this signaling axis \textit{in vivo}.

We examined mutations in all four NOTCH genes (NOTCH1-4), RB1 and TP53 by NGS in 35 SCLCs, 28 extrapulmonary SCCs, 19 pulmonary LCNECs and 33 pulmonary AdCs. All samples underwent routine IHC based diagnostics to determine cell morphology by HE stain, a pulmonary tumor origin by Thyroid Transcription Factor-1 (TTF1) and cytokeratin 7/8 (CK7/8) stain and a proliferation score by Ki-67 stain. Additionally, we included ASCL1 and DKK1 IHC for further characterization (Figs. 4 and 5).

A comprehensive list of mutations according to carcinoma subtype is given in Supporting Information Table S4. Since the activation status of NOTCH is highly dependent on the specific mutation, an additional list stating the effect of the mutation on the specific NOTCH receptor domain and available COSMIC ID is given in Supporting Information Table S5. We excluded known single nucleotide polymorphisms (SNPs) by screening SNP databases of dbSNP (NCBI) and the NHLBI GO Exome Sequencing Project (ESP) EPSS400. We included NOTCH4 isoform variants identified by our routine diagnostics pipeline.

Genetic alterations in RB1 and TP53 were characteristic for SCLCs (RB1 91.4\%, TP53 94.3\%, combined 85.7\%) and frequently found in SCCs (RB1 42.9\%, TP53 71.4\%, combined 39.3\%) and LCNECs (RB1 36.8\%, TP53 94.7\%, combined 36.8\%).

Importantly, the AdC cohort did not harbor any RB1 mutation. 42.4\% of AdCs harbored a TP53 mutation. Thus, we reconfirm that mutual inactivation of both RB1 alleles and both TP53 alleles is a hallmark of SCLC.

Only 12.1\% of AdCs expressed ASCL1 whereas all SCLCs and LCNECs were positively stained. 54.5\% of AdCs showed a strong expression of DKK1 which we observed only in 8.5\% of SCLC, SCC and LCNEC cases.

Inactivating NOTCH mutations in NOTCH1, NOTCH2 and NOTCH3 occurred in SCLC, SCC and LCNEC. The most remarkable cohort was LCNEC where no activating NOTCH mutation occurred. Thus, although NOTCH mutations were a rare genetic event, we postulate that they occur predominantly in NE lesions and that they are a hallmark of a representative subgroup of NE differentiated neoplasms including secondary SCLC that relapsed from NSCLC induced by cancer therapy (Fig. 6a).

Primary and secondary SCLC within the network of pulmonary NE lesions

Finally, we analyzed representative cases of four NE lung carcinoma categories, namely LCNEC, primary combined SCLC, NE NSCLC and secondary SCLC (Fig. 6). We performed NGS, IHC and FISH analysis if applicable (Fig. 6a and Supporting Information Fig. S7). Material of such specimens is often limited because most patients with aggressive NE lung cancers receive radio-chemotherapies and are diagnosed by small biopsies.

Case 1 was a LCNEC with a high Ki67 index (70\%) comparable to that of SCLCs. NGS revealed a NOTCH4 isoform (SNP), an inactivating NOTCH2 mutation and alterations in RB1 and TP53. Interestingly, the RB1/chromosome 13 quotient was 1.25, referring to no allelic RB1 deletion (Fig. 6a and Supporting Information Fig. S7).

Case 2 harbored combined synchronous AdC, SqCC and SCLC which were discriminated by characteristic IHC stain pattern (Supporting Information Fig. S7). The AdC harbored a KRAS mutation. All three tumor entities revealed the same NOTCH4 isoform variant and the same inactivating NOTCH3 mutation (SNPs). Importantly, only the SCLC showed an additional inactivating NOTCH2 mutation, and additional RB1 and TP53 missense mutations. RB1/chromosome 13 ratio determined in AdC and SqCC with 1.06 and 0.92, respectively, referred to no allelic RB1 deletion. In the SCLC, the ratio of 0.44 indicated heterozygous RB1 deletion. Combined FISH and NGS results indicated a complete loss of both RB1 alleles. Furthermore, we detected bi-allelic TP53 inactivation by positive p53 IHC stain and TP53 mutation with an excessively high allelic frequency of [mt]80\% (Supporting Information Table S4) suggesting presence of the mutated p53 allele and allelic loss affecting the wt allele (Fig. 6a and Supporting Information S7).

These results indicate a different tumor origin of the SCLC component compared to the AdC and the SqCC, thus representing a primary SCLC as part of a combined lung carcinoma.

Case 3 harbored two distinct but synchronous AdCs, one AdC harbored STK11 and KEAP1 mutations, the other AdC KRAS and KEAP1 mutations. The two KEAP1 mutations were not identical. Both tumors showed the same NOTCH4 isoform variant (SNP). The KRAS mutated AdC showed NE differentiation and harbored an additional inactivating NOTCH2 mutation. RB1/chromosome 13 ratio was 1.12 and 1.08, respectively, indicating no allelic RB1 deletion (Fig. 6a and Supporting Information Fig. S7). Since this NE differentiated lung carcinoma did not provide the typical rosette structure of LCNECs (Fig. S7b), this case represents a further NE lung carcinoma category of NE NSCLC.

Case 4 was defined as combined AdC with SCLC. However, this combined carcinoma was a relapse from TKI-treated AdC. We received extracts from both specimens. NGS analysis revealed an EGFR mutated AdC as primary tumor. Furthermore, this AdC harbored a TP53 mutation and an inactivating NOTCH2 mutation. Unfortunately, FFPE material was so limited that no further IHC and FISH analysis could be performed to determine NE marker expression. In the relapsed combined AdC-SCLC specimen we identified...
Figure 4. Distribution of genetic lesions in different small cell carcinoma entities. Mutations in RB1 and TP53 shown in the upper panel. Missense mutations occurring in all four NOTCH genes (NOTCH1-4) shown in the lower panel. DNA of 35 small cell lung carcinomas (SCLC) and 28 extrapulmonary small cell carcinomas (SCC) was analyzed by NGS. Organ of origin: P–Parotis; Lx–Larynx; T–Trachea; E–Esophagus; St–Stomach; Cx–Cervix; B–Bladder; U–Urothel; Pr–Prostate; Sk–Skin. – not expressed; 1 expressed; 11 strongly expressed; * dot-like expressed; na – not available.
Figure 5. Distribution of genetic lesions in different lung carcinoma entities. Mutations in RB1 and TP53 shown in the upper panel. Missense mutations occurring in all four NOTCH genes (NOTCH1-4) shown in the lower panel. DNA of 19 pulmonary large cell neuroendocrine carcinomas (LCNEC) and 33 pulmonary adenocarcinomas (AdC) was analyzed by NGS. – not expressed; + expressed; ++ strongly expressed; * dot-like expressed; na – not available.
the same EGFR mutation and the same TP53 and NOTCH2 mutation. In addition, the combined AdC SCLC harbored a RB1 splice mutant.

Allelic fractions of the TP53 and the RB1 mutation were > 90%. Thus, we postulate mutual bi-allelic alteration of both genes defined as a prerequisite for SCLC formation. As all other mutations were identical in the primary tumor and the relapse we conclude that the SCLC fraction represented a small cell outgrowth of a transdifferentiated NSCLC.

Small cell outgrowths as secondary NSCLC relapse or as primary synchronous combined carcinomas were often observed in combination with LCNEC (Supporting Information Fig. S8). For secondary SCLC, bi-allelic TP53 mutations in the non-small cell precursor may be a prerequisite, which was more frequent in SqCCs than in AdCs (Supporting Information Fig. S9). However, an independent, primary SCLC origin or a NSCLC-dependent secondary SCLC origin can only be determined by NGS.

Taken together we here established features of “small cell-ness” and confirmed the central signaling axis of NOTCH-ASCL1-RB-p53. Finally, our results provide evidence for a signaling pathway based on inactivating NOTCH mutations that drive the development of NE neoplasms including secondary SCLC (Fig. 6b).

Discussion

In this study, we comprehensively investigated features of “small cell-ness” and genetic alterations underlying the NE and SCC phenotype. We used NGS and in vitro assays to
Previous hypotheses that cytoskeletal alterations drive “small cell-ness” and epithelial to mesenchymal transition (EMT) were not confirmed, and we rather showed that these were secondary events in SCC pathology which may be controlled by NOTCH-signaling. Consistent to our features of “small cell-ness,” inactivation of all four alleles of RB1 and TP53 were described to be causative for SCC, including SCLC. We confirmed that this mechanism was likely for independent primary SCLC, and suggest an alternate pathway for secondary SCLC relapsing from NSCLC with a central driving axis including NOTCH-ASCL1-RB-p53.

A multipotent epithelial precursor was discussed as tumor cell of origin 2003 by Meuwissen et al. However, in 2011 NE precursor cells were described as the predominant origin of SCLC by Sutherland et al. Experime...
References

1. Clinical Lung Cancer Genome Project (CLCGP); Network Genomic Medicine (NGM). A genomics-classified basis of human lung tumors. *Sci Transl Med* 2013;5:209ra153.

2. Epstein JI, Amin MB, Beltran H, et al. Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. *Am J Surg Pathol* 2014;38:756–77.

3. Foulkes WD, Clarke BA, Hasselblatt M, et al. No small surprise—small-cell carcinoma of the ovary, hypercalcaemic type is a malignant rhabdoid tumour. *J Pathol* 2014;233:209–14.

4. Tudor, J, Cantley, RL, Jain, S. Primary small cell carcinoma arising from a bladder diverticulum. *J Urol* 2014;192:236–7.

5. Pavithra V, Sai Shalini CN, Priya S, et al. Small cell neuroendocrine carcinoma of the cervix: a rare entity. *J Clin Diagn Res* 2014;8:147–8.

6. Peifer M, Fernández-Cuesta L, Sos ML, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genetics* 2012;44:1104–10.

7. Meuwissen R, Linn SC, Linnoila RI, et al. Induction of small cell lung cancer by somatic inactivating of both Trp53 and Rb1 in a conditional mouse model. *Cancer Cell* 2003; 4:181–189.

8. Borges M, Linnoila RI, van de Velde HJ, et al. An achaete-scute homologue essential for neuroendocrine differentiation in the lung. *Nature* 1997;386:390–3.

9. Linnoila RI, Zhao B, DeMayo JL, et al. Constitutive achaete-scute homologue-1 promotes airway dysplasia and lung neuroendocrine tumors in transgenic mice. *Cancer Res* 2000;60:4005–9.

10. Demelash A, Rudrabhatla P, Pant HC, et al. Achaete-scute homologue-1 (ASH1) stimulates migration of lung cancer cells through Cdk5/p35 pathway. *Mol Biol Cell* 2012;23:2856–66.

11. Srirunpong V, Borges MW, Strock CL, et al. Notch signaling induces rapid degradation of achaete-scute homolog I. *Mol Cell Biol* 2002; 22:3129–39.

12. South AP, Cho RJ, Aster JC. The double-edged sword of Notch signaling and survival through cell cycle regulatory inactivation and stem cell maintenance in small cell lung cancer. *J Clin Diagn Res* 2014;8:6:236–7.

13. Goodyear S, Sharma MC. Roscovitine regulates cell cycle and proliferation of breast cancer cell lines. *Exp Mol Pathol* 2007;82:25–32.

14. Pauls K, Schorle H, Jeske W, et al. Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study. *Hum Reprod* 2006;21:397–404.

15. Bodem D, von Massenhausen A, Perner S. Analysis of receptor tyrosine kinase gene amplification on the example of FGER1. *Methods Mol Biol* 2015;1233:67–79.

16. Goodyear S, Sharma MC. Roscovitine regulates cell cycle and proliferation of breast cancer cell lines. *Exp Mol Pathol* 2007;82:25–32.

17. Pauls K, Schorle H, Jeske W, et al. Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study. *Hum Reprod* 2006;21:397–404.

18. Goodyear S, Sharma MC. Roscovitine regulates cell cycle and proliferation of breast cancer cell lines. *Exp Mol Pathol* 2007;82:25–32.