Lung Cancer 138 (2019) 102–108

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

DLL3 expression in large cell neuroendocrine carcinoma (LCNEC) and association with molecular subtypes and neuroendocrine profile

B.C.M. Hermans[a], J.L. Derks[a], E. Thunnissen[b], R.J. van Suylen[c], M.A. den Bakker[d,e], H.J.M. Groen[f], E.F. Smit[g], R.A. Damhuis[h], E.C. van den Broek[i], PALGA-group[j], A. Ruland[k], E.J.M. Speel[k,1], A.M.C. Dingemans[a,*]

[a] Department of Pulmonary Diseases, GROW school for Oncology & Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands
[b] Department of Pathology, VU University Medical Centre, Amsterdam, The Netherlands
[c] Pathology-DNA, location Jeroen Bosch Hospital, s' Hertogenbosch, The Netherlands
[d] Department of Pathology, Maasstad hospital, Rotterdam, The Netherlands
[e] Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[f] Department of Pulmonary Diseases, University of Groningen and University Medical Centre, Groningen, The Netherlands
[g] Department of Thoracic Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
[h] Department Research, Comprehensive Cancer Association, Utrecht, The Netherlands
[i] PALGA Foundation, Houten, The Netherlands
[j] PALGA-group coauthors
[k] Department of Pathology, GROW school for Oncology & Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

ARTICLE INFO

Keywords:
Large cell neuroendocrine carcinoma (LCNEC)
Lung cancer
DLL3
ASCL1
RB1

ABSTRACT

Objectives: For stage IV pulmonary large cell neuroendocrine carcinoma (LCNEC), the only therapeutic option is palliative chemotherapy. DLL3 is a new therapeutic target, which seems to be often expressed in SCLC and LCNEC. It has recently been reported that DLL3 mRNA expression is particularly upregulated in the LCNEC subgroup with *STK11/KEAP1* and *TP53* co-mutations, in contrast to lower expression levels in *RB1* and *TP53* co-mutated LCNEC. Our aim was to investigate DLL3 protein expression in stage IV LCNEC and correlate data with mutational profiles (*i.e.STK11/KEAP1/RB1*), immunostaining results (pRb, NE markers) and clinical characteristics.

Materials and Methods: Immunohistochemical analysis for DLL3 (SC16.65) and ASCL1 (SC72.201) was performed on 94 and 51 FFPE tissue sections, respectively, of pathologically reviewed stage IV LCNEC. DLL3 and ASCL1 were scored positive if ≥1% of the tumor cells showed cytoplasmic/membranous or dotlike (DLL3) or nuclear (ASCL1) immunostaining. Data were correlated with available sequencing (*TP53, RB1, STK11, KEAP1),* immunostaining (pRb, NE markers) and clinical data.

Results: DLL3 was expressed in 70/94 (74%) LCNEC, 56 (80%) of which showed cytoplasmic/membranous staining. Median H-score was 55 (interquartile range 0–160). DLL3 staining was not different in pRb immunohistochemistry negative and positive patients (DLL3+ in 53/70 (76%) *vs.* 14/21 (67%), p = 0.409) or *RB1* mutated and wildtype patients (DLL3+ in 27/34 (79%) *vs.* 23/33 (70%), p = 0.361). Nevertheless, 6/6 (100%) *STK11* mutated, 10/11 (91%) *KEAP1* mutated and 9/9 (100%) *TP53* wildtype tumors were DLL3+. Furthermore, DLL3 expression was associated with expression of ASCL1 and at least 2 out of 3 neuroendocrine markers.

Conclusion: The high percentage (74%) of DLL3 expression in stage IV LCNEC denotes the potential of DLL3 targeted therapy in this patient group.

1. Introduction

Large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) are aggressive neuroendocrine tumors with poor survival rates [1–3]. For stage IV SCLC, treatment has not advanced significantly over the last decades and consists of palliative

* Corresponding author: Department of Pulmonology, Maastricht University Medical Centre, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands.
E-mail address: a.dingemans@erasmusmc.nl (A.M.C. Dingemans).
[1] Dr. Speel and dr. Dingemans contributed equally to this work.

https://doi.org/10.1016/j.lungcan.2019.10.010
Received 6 August 2019; Received in revised form 5 October 2019; Accepted 8 October 2019

chemotherapy. The same applies to stage IV LCNEC, were no standard treatment exists and palliative chemotherapy with SCLC and non-small cell lung cancer (NSCLC) regimens are both deemed appropriate [4]. Recently, targeted therapy focusing on delta like protein 3 (DLL3) has received attention to improve outcomes for SCLC and LCNEC [5].

DLL3 is part of the Notch family including four Notch receptors (Notch1-4) and five transmembrane ligands, coded by jagged (JAG1 and JAG2) and delta-like (DLL1, DLL3 and DLL4) gene families. DLL3 is an important link in the achaete-scute complex-like 1 (ASCL1) – DLL3 – Notch1 pathway. The supposed exclusive function of DLL3 is inhibition of Notch1, in contrast to DLL1 and DLL4 which have both inhibitory and stimulatory effects on the Notch pathway [6–8]. DLL3 has been reported to be a downstream target of ASCL1, a transcription factor critical for development of pulmonary neuroendocrine cells in the developing lung [9,10]. Therefore, activation of ASCL1 will result in DLL3 upregulation and increased inhibition of Notch1 [5]. In addition, Notch1 has been described to be a negative regulator of ASCL1 [11,12]. Apparently, the ASCL1 – DLL3 – Notch1 pathway is non-linear, and a change in expression of one of the proteins influences the others. In some tumor types, Notch pathway activation (*i.e.* Notch1 upregulation or ASCL1 downregulation) results in oncogenic stimulation and tumor growth [13,14]. However, both Notch1 inhibition and ASCL1 upregulation have shown to result in development of neuroendocrine neoplasms [9,14–16].

Two main molecular subtypes of LCNEC have been identified by next generation sequencing (NGS) studies. The first subtype has mutations in *TP53* and *STK11* and/or *KEAP1* (NSCLC-like), whereas the second subtype has mutations of *TP53* and *RB1* (a hallmark of SCLC) [17–19]. The subclassification could also be made based on pRb immunohistochemistry (IHC) expression, classifying tumors with loss of pRb as SCLC-like [20]. A small subset of LCNEC with carcinoid-like features has also been identified, enriched for *MEN1* mutations [17]. Our recent study emphasized clinical relevance of the two main LCNEC subtypes, by showing a worse survival for NSCLC-like LCNEC patients treated with platinum-etoposide compared to NSCLC-regimen whereas no difference was found for the SCLC-like subtype [20]. Interestingly, a recent study identified that *STK11/KEAP1* mutated LCNEC have a $Notch1^{Low}$/ $DLL3^{High}$/$ASCL1^{High}$ RNA expression signature while the *TP53/RB1* mutated LCNEC had a $Notch1^{High}$/$DLL3^{Low}$/$ASCL1^{Low}$ signature [18]. Hence, LCNEC molecular subtypes may be related to DLL3 expression also at protein level.

Only limited data on prevalence of DLL3 and no data on type of staining, percentage of positive cells within each sample or survival related to DLL3 expression in LCNEC is available. In this study, we assessed DLL3 expression by IHC in a cohort of 94 patients with well characterized and molecular profiled stage IV LCNEC. In addition, the association of DLL3 status with mutational status (*RB1, TP53, KEAP1, STK11*) and IHC expression of pRb, ASCL1, TTF1 and neuroendocrine markers was investigated.

2. Materials and method

2.1. Patient selection

All data for this retrospective population-based study were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA) as described before (2003–2012) [21,22]. Clinical data was updated until 2015 and comprised age, gender, overall survival (OS) and progression free survival (PFS).

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043).

2.2. Pathologic material

Panel consensus pathology revision was performed as described earlier for 232 stage IV LCNEC. Samples were evaluated for neuroendocrine morphology (organoid nesting, palisading, rosettes or trabeculae), mitotic index, necrosis, and neuroendocrine differentiation using IHC for at least one neuroendocrine marker (NE-marker). Diagnosis was confirmed in patients meeting the WHO-criteria [23]. If strict WHO-criteria were not met, but the pathologists found it likely that LCNEC was the correct diagnosis, an exception was made as described earlier [24,25].

IHC for pRb (13A10) were available for the majority of the patients [20]. Furthermore, for most patients, IHC results for TTF1 and NE-markers (CD56, chromogranin A and synaptophysin) were present. In case of absence of one or two of the NE-markers, extra immunostaining was performed if tissue was available. NE-markers were scored as negative, weakly (+), moderately (++) or strongly (+++) positive, as described before [25].

2.3. Immunohistochemistry

2.3.1. DLL3

We performed DLL3 immunostaining on tumors with confirmed LCNEC diagnosis if FFPE blocks with sufficient available tumor tissue were available. IHC was performed on 3 µm thick tissue sections using SC16.65 antibody (3 µg/ml) and a IHC DAKO FLEX Mouse linker protocol (both provided by Abbvie). A low pH antigen retrieval was used. A positive control (HEK-293 T.hDLL3) and a negative control (HEK-293 T) were included (also provided by Abbvie) [5]. Immunostainings were evaluated by two investigators (BH and EJS), who were blinded for all clinical, histopathological and mutational data. Selected samples were discussed with K. Isse (pathologist, Abbvie) for confirmation. Samples were scored as positive (≥ 1% positive tumor cells) or negative (< 1%) for DLL3 [26]. Scoring for positive samples was further specified by intensity (low (1), intermediate (2) or high (3)), percentage of positive cells and type of staining: more diffuse staining of both cytoplasm and membrane was scored as cytoplasmic/membranous (Fig. 1A), and a more punctuated perinuclear pattern was scored as dotlike (Fig. 1B). H-score was calculated per case by multiplying intensity with percentage of positive cells.

2.3.2. ASCL1

IHC for ASCL1 was performed if sufficient tumor material was available. IHC was performed on 3 µm thick slides using SC72.201 antibody (1 µg/ml) and the IHC DAKO FLEX + mouse linker protocol (both provided by Abbvie). A low pH antigen retrieval was used. An ASCL1 positive cell line and SCLC sample were used as positive

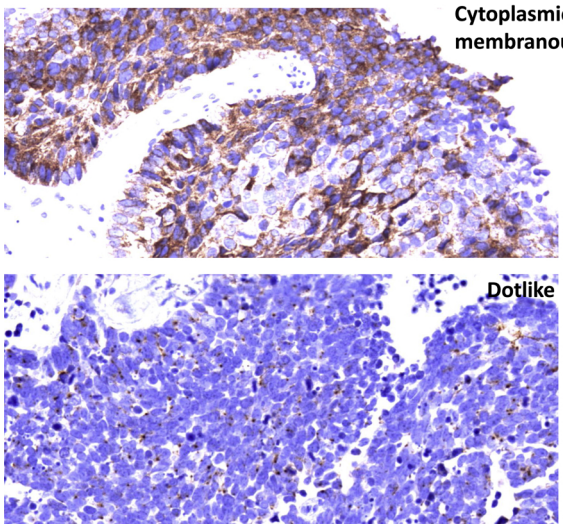

Fig. 1. Representative samples of DLL3 immunohistochemistry on LCNEC tumors. A) Combined cytoplasmic and membranous staining. B) Perinuclear dotlike staining.

controls. The negative controls were an ASCL1 negative cell line and an SCLC sample. Nuclear staining was evaluated by two investigators (BH and EJS), who were blinded for all clinical, histopathological and mutational data. Samples were scored positive (≥1% positive tumor cells) or negative (< 1%). Scoring for positive samples was further specified by intensity (low (1), intermediate (2) or high (3)) and percentage of positive cells. H-score was calculated per case by multiplying intensity with percentage of positive cells.

2.4. Mutational analysis

DNA was isolated from available FFPE tissue blocks and targeted NGS was performed for *RB1*, *KEAP1*, *STK11* and *TP53*, as described earlier [20]. All co-mutated *RB1* and *TP53* samples as well as pRb IHC negative/*TP53* mutated samples were classified as SCLC-like. All other samples were classified as non SCLC-like. Since the third LCNEC subgroup with carcinoid-like morphology and *MEN1* mutations is very small, this subgroup was not further addressed in this study.

2.5. Statistics

All analyses were performed using SPSS (version 25 for Windows, Armonk, NY: IBM Corp.). Association of DLL3 status (DLL3+ or DLL3-) with gender, mutational status (*RB1, STK11, KEAP1* and *TP53* mutation), number of positive NE-markers (1 or ≥2) and positive immunostaining for pRb, TTF1 and ASCL1, was investigated with Chi-squared test or Fisher's Exact Test. Association between DLL3 H-score and ASCL1 H-score was investigated with Spearman correlation. Differences in median age in DLL3+ *versus* DLL3- patients and differences in median DLL3 H-score in patients with 1 NE-marker *versus* ≥2 NE-markers were tested with Mann Whitney U test. Differences in median DLL3 H-score for different intensities of NE-markers were analyzed with Kruskal-Wallis test. Median overall survival (OS) was evaluated by Kaplan Meier analysis and differences in survival were tested for significance with Log-Rank test for DLL3 positive and negative staining. Results are presented as hazard ratios (HR) with 95% confidence intervals (CI). P < 0.05 was considered significant.

3. Results

3.1. Patient characteristics

DLL3 immunostaining was performed in 94 out of 148 patients with consensus based confirmed LCNEC *(Supplemental figure A)*. Mean age at diagnosis was 63 years (range 34–82 years). A total of 61% of patients were male (Table 1). Staining for three NE-markers (CD56, synaptophysin, chromogranin A), pRb and TTF1 was available in 91, 91 and 83 samples, respectively. NGS for *STK11*, *KEAP1, RB1* and *TP53* was available for 67 patients (*Supplemental figure A*). For ASCL1 IHC, only in 51 cases sufficient tumor tissue was available.

3.2. DLL3 IHC

DLL3 staining was positive (≥1% of tumor cells positive) in 70/94 (74%) samples (Table 1). Of the 94 patients, 62 (66%) had DLL3 staining in ≥25% of tumor cells, 51 (54%) in ≥50% and 35 (37%) in ≥75%. Median H-score was 55 (interquartile range (IQ) 0–160). Of the 70 DLL3 positive samples, 56 (80%) had mainly cytoplasmic/membranous staining and only 14 (20%) had mainly perinuclear dotlike staining (Table 1, Fig. 1). Isolated membranous staining was not observed. DLL3 expression was not associated with gender or age (Table 1). A trend towards more DLL3 positivity in TTF1 positive LCNEC compared to TTF1 negative LCNEC was observed (52/63 (83%) *vs.* 12/20 (60%), p = 0.063) (Fig. 2, *Supplemental table A*). OS in DLL3+ patients was comparable to DLL3- patients (6.9 months (95% CI 5.1–8.7) *vs.* 6.1 months (95% CI 4.3–7.9), HR 1.00, p = 1.00) *(Supplemental figure B)*.

Table 1
DLL3 expression in stage IV LCNEC.

	DLL3+	DLL3-	P-value
Total (N = 94)	70 (74%)	24 (26%)	
% positive cells			
≥1 %	70 (74%)	–	
≥25%	62 (66%)	–	
≥50%	51 (54%)	–	
≥75%	35 (37%)	–	
H-score (N = 70)			
≤100	33 (47%)	–	
101-≤200	21 (30%)	–	
201-≤300	16 (23%)	–	
Type of staining (N = 70)			
Mainly dotlike	14 (20%)	–	
Mainly cytoplasmic/ membranous	56 (80%)	–	
Patient characteristics			
Gender			
Male (N = 57)	44 (77%)	13 (23%)	0.45*
Female (N = 37)	26 (70%)	11 (30%)	
Age (median, IQ range)	62 (55-71)	65 (60-71)	0.28**

IQ range = interquartile range.
* Chi-square.
** Mann-Whitney U test.

3.3. DLL3 in relation to LCNEC mutational subtypes

No difference was found between pRb IHC positive and negative groups (DLL3+ in 14/21 (67%) *vs.* 53/70 (76%), p = 0.41) (Fig. 2, *Supplemental table A*). Also, no difference was found between *RB1* wildtype and *RB1* mutated subgroups (DLL3+ in 23/33 (70%) *vs.* 27/34 (79%), p = 0.36). After classification of samples by combining information on *TP53* and *RB1* mutation and pRb IHC expression, 67 SCLC-like and 24 non SCLC-like cases were identified. No difference in DLL3 expression was found in the two subgroups (DLL3+ in 49/67 (73%) *vs.* 18/24 (75%), p = 0.86). Regarding additional mutational analysis, 6/6 (100%) *STK11* mutated *vs.* 44/61 (72%) *STK11* wildtype (p = 0.33) and 10/11 (91%) *KEAP1* mutated *vs.* 40/56 (71%) *KEAP1* wildtype tumors (p = 0.27) were DLL3 positive. Furthermore, 9/9 (100%) *TP53* wildtype tumors were DLL3 positive *vs.* 41/58 (71%) *TP53* mutated tumors (p = 0.098). No differences were found for the type of staining in mutational subtypes (data not shown). In case a cut-off value of ≥50% was used for DLL3 positivity, only for *TP53* wildtype tumors compared to *TP53* mutated tumors a significantly higher DLL3 expression was found (DLL3 ≥ 50% in 8/9 (89%) *vs.* 29/58 (50%), p = 0.035) *(Supplemental table B)*.

3.4. DLL3 in relation to neuroendocrine marker profile

In tumors with ≥2 positive NE-markers, DLL3 was expressed significantly more often compared to tumors with 1 positive NE-marker (DLL3+ in 66/82 (81%) *vs.* 3/9 (33%), p = 0.006), and median DLL3 H-score was higher in the group with ≥2 positive NE-markers (77.5 (IQ 18–160) *vs.* 0 (IQ 0–40), p = 0.02) (Fig. 3). Remarkably, 3/3 (100%) DLL3+ patients with only 1 positive NE-marker had dotlike staining, while in the samples with ≥2 positive NE-markers the fraction of dotlike staining was 11/56 (20%) (Fig. 3). Furthermore, an increased median DLL3 H-score was associated with an increased staining intensity of synaptophysin and chromogranin A, but not with CD56 (Table 2, *Supplemental figure C*). Percentage of DLL3+ patients was higher in the ASCL1 positive group compared to the ASCL1 negative group (DLL3+ in 35/39 (90%) *vs.* 6/12 (50%), p = 0.007) (Fig. 2, *Supplemental table A*). Furthermore, DLL3 H-score and ASCL1 H-score were correlated (Spearman correlation 0.38, p = 0.007) (*Supplemental figure D*).

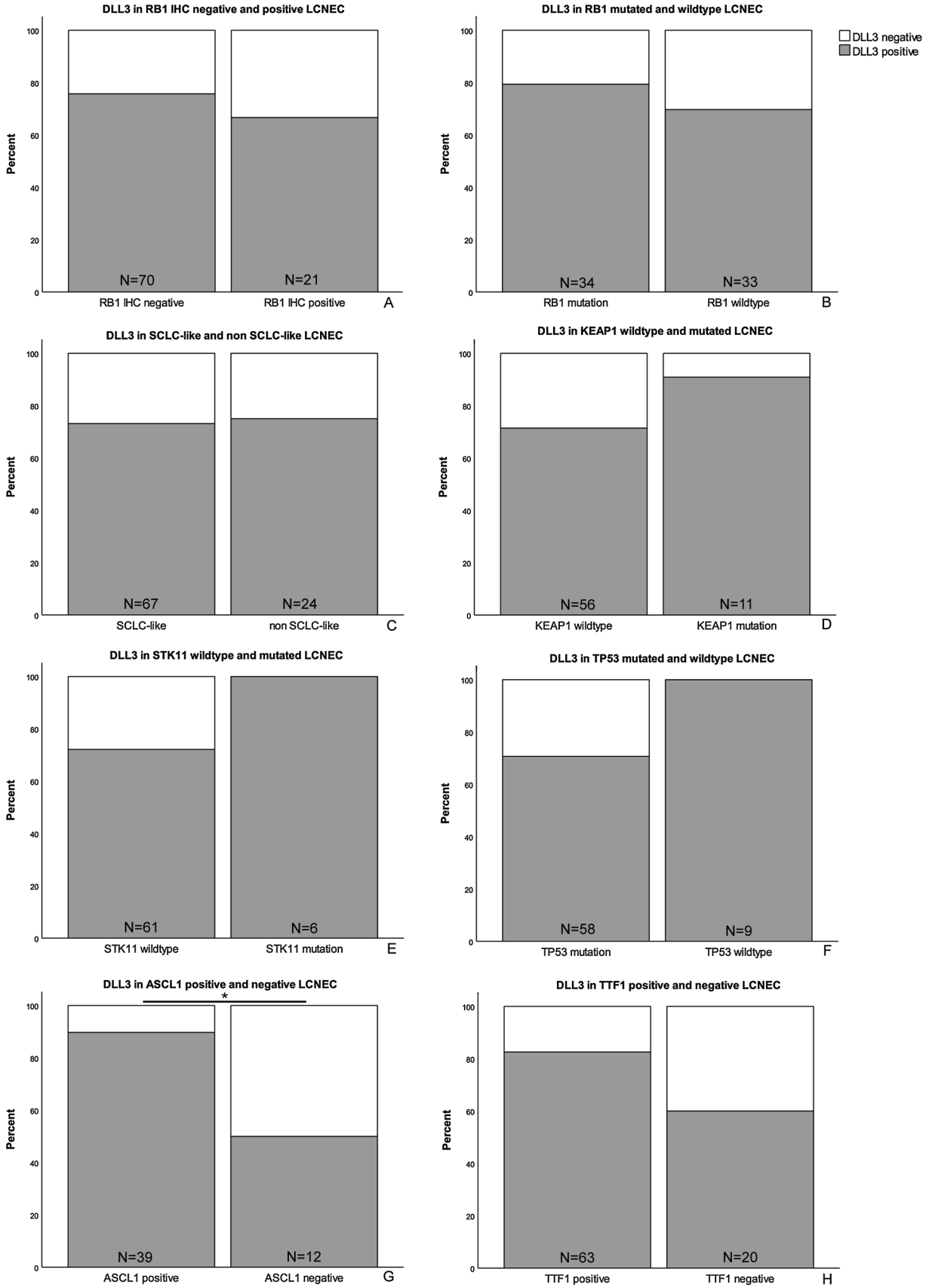

Fig. 2. DLL3 expression in LCNEC: A) pRb IHC negative (N = 70) & pRb IHC positive (N = 21) B) RB1 mutated (N = 34) & RB1 wildtype (N = 33) C) SCLC-like (N = 67) & non SCLC-like (N = 24) D) KEAP1 wildtype (N = 56) & KEAP1 mutated (N = 11) E) STK11 wildtype (N = 61) & STK11 mutated (N = 6) F) TP53 mutated (N = 58) & TP53 wildtype (N = 9) G) ASCL1 IHC positive (N = 39) & ASCL1 IHC negative (N = 12) H) TTF1 IHC positive (N = 63) & TTF1 IHC negative (N = 20). *Statistically significant (Fisher's Exact Test).

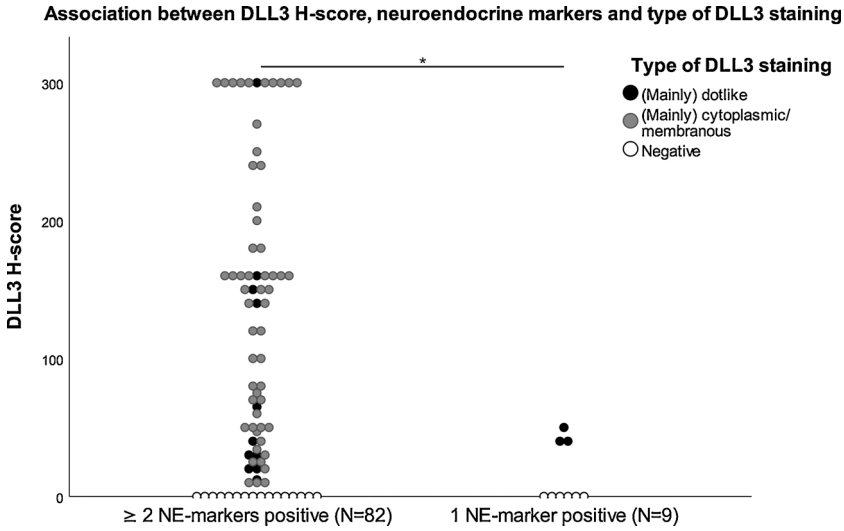

Fig. 3. DLL3 H-score and type of DLL3 staining in tumors with ≥2 positive NE-markers (median H-score 77.5) and 1 positive NE-marker (median H-score 0, p = 0.002 (Mann-Whitney U test)).
NE-marker = neuroendocrine marker, = Median H-score

Table 2
Correlation between DLL3 H-score and staining intensity of neuroendocrine markers.

Staining intensity	Median DLL3 H-score (IQ range)	p-value
Chromogranin A		
Neg	40 (0–73)	0.015*
+	85 (0–185)	
+ +	65 (20–160)	
+ + +	160 (50–300)	
Synaptophysin		
Neg	0 (0–20)	0.001*
+	80 (0–170)	
+ +	41 (0–135)	
+ + +	150 (43–263)	
CD56		
Neg	44 (8–150)	0.55*
+	110 (24–243)	
+ +	50 (0–160)	
+ + +	55 (0–160)	

IQ range = interquartile range.
+ = low intensity, + + = intermediate intensity, + + + = high intensity.
* Kruskal-Wallis test.

In both subgroups with (almost) 100% of samples expressing DLL3 (*STK11/KEAP1* mutated and *TP53* wildtype), 100% of samples were positive for ≥2 NE-markers. On the contrary, in all 91 patients with all NE-markers performed, ≥2 NE-markers were positive in only 82 samples (90%). ASCL1 was also highly expressed in the *STK11/KEAP1* mutated group, whereas an unexpected trend for low ASCL1 expression was seen in the *TP53* wildtype group compared to *TP53* mutated group (ASCL1+ in 4/8 (50%) *vs.* 33/41 (81%), p = 0.088).

4. Discussion

In this study, we found a high prevalence of DLL3 positivity in stage IV LCNEC and demonstrated that DLL3 is especially high in *STK11* and *KEAP1* mutated or *TP53* wildtype tumors and in tumors positive for ASCL1 and ≥2 neuroendocrine markers. The prevalence of DLL3 expression in LCNEC is comparable to SCLC and might therefore also be a potential therapeutic target in LCNEC.

We demonstrated DLL3 expression in 74% of 94 stage IV LCNEC patients, comparable with the only previous study in LCNEC reporting positive immunohistochemical DLL3 staining in 37/57 (65%) of samples [5]. So far, no data on type of staining, percentage of positive cells within each sample, or survival related to DLL3 expression was available for LCNEC. In the present study, DLL3 was expressed in ≥50% of tumor cells in the majority of samples (54%). Reported percentages of DLL3 expression for SCLC are slightly higher (72–90%), with the majority of positive samples having a high percentage of tumor cells (≥50%) expressing DLL3 [5,26–28]. The majority of LCNEC in our study had cytoplasmic and membranous staining, as was reported before in SCLC [5,28]. DLL3 expression did not correlate with prognosis in this LCNEC cohort. The only study evaluating survival in DLL3+ and DLL3- SCLC patients without DLL3 targeted treatment, demonstrated similar results [28].

DLL3 expression has been related with mutational status and expression profiles of ASCL1 and Notch1 in LCNEC. George et al. found an $ASCL1^{High}$/ $DLL3^{High}$/ $Notch^{Low}$ gene expression profile and high expression levels of neuroendocrine genes (synaptophysin, chromogranin A) in LCNEC with *TP53* and *STK11/KEAP1* mutations [18]. On the other hand, in LCNEC with *TP53* and *RB1* mutations, an $ASCL1^{Low}$/ $DLL3^{Low}$/ $Notch^{High}$ gene expression profile and lower expression levels of neuroendocrine genes were found [18]. In accordance with this study, we found all *STK11* mutated and 10/11 *KEAP1* mutated samples to be immune positive for DLL3. Furthermore, a high percentage of those tumors had ASCL1 expression and all had ≥2 NE-markers positive. However, we did not find any relation with *RB1* mutation status or pRb IHC staining. In addition, a special subgroup of LCNEC, wildtype for *TP53*, with an $ASCL1^{Low}$/ $DLL3^{High}$ profile, was identified. Since this study comprises only a limited number of patients in each subgroup, further research is necessary to verify DLL3 and ASCL1 expression in these subgroups.

A correlation between TTF1 and DLL3 expression in SCLC was found by Cardnell et al., suggesting that TTF1 could be used as a surrogate marker for DLL3 [29]. We could not confirm this correlation and in our study 28% of tumors would be misclassified as DLL3 IHC positive or negative if TTF1 would be used as a surrogate marker for DLL3.

Recently four subtypes of SCLC were defined by expression of ASCL1, NEUROD1, POU2F3 and YAP1 [30]. Only the first group with ASCL1 expression, the classic SCLC, was found to have high DLL3 expression, whereas the other smaller groups had no or limited expression of DLL3 and ASCL1 [30]. In future research, NEUROD1, POU2F3 and YAP1 could also be tested in LCNEC and correlated to ASCL1 and DLL3 expression.

This study has some limitations. Since it is a retrospective study, not

all clinical characteristics (*i.e.* smoking history) could be obtained. Also, material was not sufficient in all patients to perform NGS, evaluate NE-markers and perform IHC for pRb, DLL3 and ASCL1. Though clear distinction between dotlike and cytoplasmic staining could be made, discrimination between cytoplasmic staining only and combined cytoplasmic and membranous staining was not possible. Therefore, all cytoplasmic stained samples are considered to have membranous staining as well. Former studies also found a combined cytoplasmic/membranous staining in the majority of tumors [5,28]. So far, it is not known whether type of staining predicts response to DLL3 targeted therapy. Furthermore, it is not yet known if the cut-off value of ≥1% is clinically relevant or that a higher cut-off value should be chosen. One clinical study found improved outcomes in patients with high DLL3 expression (≥50%) compared to low DLL3 expression (≥1-50%), whereas preliminary results of another study did not find a difference between high (≥75%) and low (≥25-75%) DLL3 expression [26,31]. Finally, we used the mouse DLL3 antibody (clone SC16.65) in this study whilst other studies use the rabbit antibody (SP347). So far, no reports are published comparing these two antibodies.

The high percentage of DLL3 positive SCLC and LCNEC combined with low or non-detectable DLL3 levels in healthy tissue, make DLL3 attractive for targeted therapy [5,27,28]. In normal tissue, DLL3 mRNA is only expressed within the brain and in very low amounts within esophagus and pancreas [5,18]. The first-in-class drug to target DLL3 expressing tumors is an antibody-drug conjugate: rovalpituzumab-tesirine (Rova-T) [32]. After promising results in patient derived xenograft (PDX) mice models and a phase 1 study with Rova-T, several clinical trials were initiated for patients with SCLC and other solid (neuroendocrine) tumors, including LCNEC [5,26]. Unfortunately, a phase 2 trial (TRINITY) found a response in only a limited number of patients and interim analysis by the Independent Data Monitoring Committee of two phase 3 studies (TAHOE and MERU) revealed lack of survival benefit in the Rova-T arm compared to the control arm [33–35]. Both studies were closed for inclusion and development of Rova-T was halted by Abbvie [34,35].

Two other approaches of targeting DLL3 are a bi-specific T-cell engager (BiTE®) antibody construct (AMG 757 and DLL3/CD3 ITE) and adoptive chimeric antigen receptor T-cell (CAR-T) therapy (AMG 119). Preclinical studies showed a good safety profile and phase I trials are currently enrolling (NCT03319940 and NCT03392064, respectively) [36,37]. Hopefully, these new approaches will be more successful than Rova-T in targeting DLL3 and treating SCLC and LCNEC.

In this study we demonstrated a high prevalence of cytoplasmic/membranous DLL3 positivity in patients with stage IV LCNEC. This high DLL3 percentage in LCNEC calls for further study of recently developed DLL3 targeting agents such as approaches with BiTE® and CAR-T.

Funding source

AbbVie (Quebec, Canada) provided DLL3 and ASCL1 antibodies and immunostaining protocols for this study. Immunostaining of selected tumor samples was discussed with an expert pathologist of AbbVie. AbbVie agreed with submission of the paper.

Conflict of interest statement

Dr. Hermans reports non-financial support from Abbvie, during the conduct of the study; grants from Bristol Myers-Squibb, outside the submitted work;

Dr. Derks reports non-financial support from Abbvie, during the conduct of the study; personal fees from IPSEN, Boehringher-Ingelheim, Pfizer and BMS, grants from Bristol-Myers Squibb,outside the submitted work;

Dr. Groen reports grants from AbbVie (participation in clinical trials), grants and other from Eli Lilly, Roche, Merck, BMS, Novartis, outside the submitted work;

Dr. Speel reports non-financial support from Abbvie, during the conduct of the study; grants from Astra Zeneca, Pfizer, Novartis, MSD and Bristol-Myers Squibb, personal fees from AbbVie and Roche, outside the submitted work;

Dr. Dingemans reports non-financial support from Abbvie, during the conduct of the study; personal fees from Roche, BMS, Eli Lily and Takeda Boehringer Ingelheim, grants from Bristol-Myers Squibb, outside the submitted work;

The other authors did not report conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.10.010.

References

[1] J.L. Derks, L.E. Hendriks, W.A. Buikhuisen, H.J. Groen, E. Thunnissen, R.J. van Suylen, R. Houben, R.A. Damhuis, E.J. Speel, A.M. Dingemans, Clinical features of large cell neuroendocrine carcinoma: a population-based overview, Eur. Respir. J. 47 (2) (2016) 615–624.
[2] H. Asamura, T. Kameya, Y. Matsuno, M. Noguchi, H. Tada, Y. Ishikawa, T. Yokose, S.X. Jiang, T. Inoue, K. Nakagawa, K. Tajima, K. Nagai, Neuroendocrine neoplasms of the lung: a prognostic spectrum, J. Clin. Oncol. 24 (1) (2006) 70–76.
[3] G. Rossi, A. Cavazza, A. Marchioni, L. Longo, M. Migaldi, G. Sartori, N. Bigiani, L. Schirosi, C. Casali, U. Morandi, N. Facciolongo, A. Maiorana, M. Bavieri, L.M. Fabbri, E. Brambilla, Role of chemotherapy and the receptor tyrosine kinases KIT, PDGFRalpha, PDGFRbeta, and Met in large-cell neuroendocrine carcinoma of the lung, J. Clin. Oncol. 23 (34) (2005) 8774–8785.
[4] G.A. Masters, S. Temin, C.G. Azzoli, G. Giaccone, S. Baker Jr., J.R. Brahmer, P.M. Ellis, A. Gajra, N. Rackear, J.H. Schiller, T.J. Smith, J.R. Strawn, D. Trent, D.H. Johnson, American society of clinical oncology clinical, systemic therapy for stage IV non-small-Cell lung Cancer: american society of clinical oncology clinical practice guideline update, J. Clin. Oncol. 33 (30) (2015) 3488–3515.
[5] L.R. Saunders, A.J. Bankovich, W.C. Anderson, M.A. Aujay, S. Bheddah, K. Black, R. Desai, P.A. Escarpe, J. Hampl, A. Laysang, D. Liu, J. Lopez-Molina, M. Milton, A. Park, M.A. Pysz, H. Shao, B. Slingerland, M. Torgov, S.A. Williams, O. Foord, P. Howard, J. Jassem, A. Badzio, P. Czapiewski, D.H. Harpole, A. Dowlati, P.P. Massion, W.D. Travis, M.C. Pietanza, J.T. Poirier, C.M. Rudin, R.A. Stull, S.J. Dylla, A DLL3-targeted antibody-drug conjugate eradicates high-grade pulmonary neuroendocrine tumor-initiating cells in vivo, Sci. Transl. Med. 7 (302) (2015) 302ra136.
[6] A. Ayyanan, G. Civenni, L. Ciarloni, C. Morel, N. Mueller, K. Lefort, A. Mandinova, W. Raffoul, M. Fiche, G.P. Dotto, C. Brisken, Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism, Proc Natl Acad Sci U S A 103 (10) (2006) 3799–3804.
[7] S.M. Deng, X.C. Yan, L. Liang, L. Wang, Y. Liu, J.L. Duan, Z.Y. Yang, T.F. Chang, B. Ruan, Q.J. Zheng, H. Han, The Notch ligand delta-like 3 promotes tumor growth and inhibits Notch signaling in lung cancer cells in mice, Biochem. Biophys. Res. Commun. 483 (1) (2017) 488–494.
[8] G. Chapman, D.B. Sparrow, E. Kremmer, S.L. Dunwoodie, Notch inhibition by the ligand DELTA-LIKE 3 defines the mechanism of abnormal vertebral segmentation in spondylocostal dysostosis, Hum. Mol. Genet. 20 (5) (2011) 905–916.
[9] T. Jiang, B.J. Collins, N. Jin, D.N. Watkins, M.V. Brock, W. Matsui, B.D. Nelkin, D.W. Ball, Achaete-scute complex homologue 1 regulates tumor-initiating capacity in human small cell lung cancer, Cancer Res. 69 (3) (2009) 845–854.
[10] R.M. Henke, D.M. Meredith, M.D. Borromeo, T.K. Savage, J.E. Johnson, Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube, Dev. Biol. (Basel) 328 (2) (2009) 529–540.
[11] E.K. Nakakura, V.R. Sriuranpong, M. Kunnimalaiyaan, E.C. Hsiao, K.E. Schuebel, M.W. Borges, N. Jin, B.J. Collins, B.D. Nelkin, H. Chen, D.W. Ball, Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by notch signaling, J. Clin. Endocrinol. Metab. 90 (7) (2005) 4350–4356.
[12] V. Sriuranpong, M.W. Borges, C.L. Strock, E.K. Nakakura, D.N. Watkins, C.M. Blaumueller, B.D. Nelkin, D.W. Ball, Notch signaling induces rapid degradation of achaete-scute homolog 1, Mol. Cell. Biol. 22 (9) (2002) 3129–3139.
[13] K. Maemura, H. Yoshikawa, K. Yokoyama, T. Ueno, H. Kurose, K. Uchiyama, Y. Otsuki, Delta-like 3 is silenced by methylation and induces apoptosis in human hepatocellular carcinoma, Int. J. Oncol. 42 (3) (2013) 817–822.
[14] I. Espinoza, L. Miele, Notch inhibitors for cancer treatment, Pharmacol. Ther. 139 (2) (2013) 95–110.
[15] M. Kunnimalaiyaan, H. Chen, Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors, Oncologist 12 (5) (2007) 535–542.
[16] R.I. Linnoila, B. Zhao, J.L. DeMayo, B.D. Nelkin, S.B. Baylin, F.J. DeMayo, D.W. Ball, Constitutive achaete-scute homologue-1 promotes airway dysplasia and lung neuroendocrine tumors in transgenic mice, Cancer Res. 60 (15) (2000) 4005–4009.
[17] N. Rekhtman, M.C. Pietanza, M.D. Hellmann, J. Naidoo, A. Arora, H. Won, D.F. Halpenny, H. Wang, S.K. Tian, A.M. Litvak, P.K. Paik, A.E. Drilon, N. Socci, J.T. Poirier, R. Shen, M.F. Berger, A.L. Moreira, W.D. Travis, C.M. Rudin,

M. Ladanyi, Next-generation sequencing of pulmonary large cell neuroendocrine carcinoma reveals small cell carcinoma-like and non-small cell carcinoma-like subsets, Clin. Cancer Res. 22 (14) (2016) 3618–3629.
[18] J. George, V. Walter, M. Peifer, L.B. Alexandrov, D. Seidel, F. Leenders, L. Maas, C. Muller, I. Dahmen, T.M. Delhomme, M. Ardin, N. Leblay, G. Byrnes, R. Sun, A. De Reynies, A. McLeer-Florin, G. Bosco, F. Malchers, R. Menon, J. Altmuller, C. Becker, P. Nurnberg, V. Achter, U. Lang, P.M. Schneider, M. Bogus, M.G. Soloway, M.D. Wilkerson, Y. Cun, J.D. McKay, D. Moro-Sibilot, C.G. Brambilla, S. Lantuejoul, N. Lemaitre, A. Soltermann, W. Weder, V. Tischler, O.T. Brustugun, M. Lund-Iversen, A. Helland, S. Solberg, S. Ansen, G. Wright, B. Solomon, L. Roz, U. Pastorino, I. Petersen, J.H. Clement, J. Sanger, J. Wolf, M. Vingron, T. Zander, S. Perner, W.D. Travis, S.A. Haas, M. Olivier, M. Foll, R. Buttner, D.N. Hayes, E. Brambilla, L. Fernandez-Cuesta, R.K. Thomas, Integrative genomic profiling of large-cell neuroendocrine carcinomas reveals distinct subtypes of high-grade neuroendocrine lung tumors, Nat. Commun. 9 (1) (2018) 1048.
[19] J.L. Derks, N. Leblay, S. Lantuejoul, A.C. Dingemans, E.M. Speel, L. Fernandez-Cuesta, New insights into the molecular characteristics of pulmonary carcinoids and large cell neuroendocrine carcinomas, and the impact on their clinical management, J. Thorac. Oncol. 13 (6) (2018) 752–766.
[20] J.L. Derks, N. Leblay, E. Thunnissen, R.J. van Suylen, M. den Bakker, H.J.M. Groen, E.F. Smit, R. Damhuis, E.C. van den Broek, A. Charbrier, M. Foll, J.D. McKay, L. Fernandez-Cuesta, E.M. Speel, A.C. Dingemans, P. Group, Molecular subtypes of pulmonary large-cell neuroendocrine carcinoma predict chemotherapy treatment outcome, Clin. Cancer Res. 24 (1) (2018) 33–42.
[21] J.L. Derks, R.J. van Suylen, E. Thunnissen, M.A. den Bakker, E.F. Smit, H.J. Groen, E.J. Speel, A.M. Dingemans, A population-based analysis of application of WHO nomenclature in pathology reports of pulmonary neuroendocrine tumors, J. Thorac. Oncol. 11 (4) (2016) 593–602.
[22] M. Casparie, A.T. Tiebosch, G. Burger, H. Blauwgeers, A. van de Pol, J.H. van Krieken, G.A. Meijer, Pathology databanking and biobanking in the Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive, Cell. Oncol. 29 (1) (2007) 19–24.
[23] W.D. Travis, E. Brambilla, A.P. Burke, A. Markx, A.G. Nicholson, WHO classification of tumours of the lung, pleura, Thymus and Heart, fourth ed., International Agency for Research on Cancer, Lyon, France, 2015.
[24] J.L. Derks, R.J. van Suylen, E. Thunnissen, M.A. den Bakker, H.J. Groen, E.F. Smit, R.A. Damhuis, E.C. van den Broek, E.M. Speel, A.C. Dingemans, P. group, Chemotherapy for pulmonary large cell neuroendocrine carcinomas: does the regimen matter? Eur. Respir. J. 49 (6) (2017).
[25] J.L. Derks, A.C. Dingemans, R.J. van Suylen, M.A. den Bakker, R.A.M. Damhuis, E.C. van den Broek, E.J. Speel, E. Thunnissen, Is the sum of positive neuroendocrine immunohistochemical stains useful for diagnosis of large cell neuroendocrine carcinoma (LCNEC) on biopsy specimens? Histopathology 74 (4) (2019) 555–566.
[26] C.M. Rudin, M.C. Pietanza, T.M. Bauer, N. Ready, D. Morgensztern, B.S. Glisson, L.A. Byers, M.L. Johnson, H.A. Burris 3rd, F. Robert, T.H. Han, S. Bheddah, N. Theiss, S. Watson, D. Mathur, B. Vennapusa, H. Zayed, S. Lally, D.K. Strickland, R. Govindan, S.J. Dylla, S.L. Peng, D.R. Spigel, S. investigators, Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study, Lancet Oncol. 18 (1) (2017) 42–51.
[27] M. Saito, K. Saito, K. Shiraishi, D. Maeda, H. Suzuki, Y. Minamiya, K. Kono, T. Kohno, A. Goto, Identification of candidate responders for anti-PD-L1/PD-1 immunotherapy, Rova-T therapy, or EZH2 inhibitory therapy in small-cell lung cancer, Mol. Clin. Oncol. 8 (2) (2018) 310–314.
[28] K. Tanaka, K. Isse, T. Fujihira, M. Takenoyama, L. Saunders, S. Bheddah, Y. Nakanishi, I. Okamoto, Prevalence of Delta-like protein 3 expression in patients with small cell lung cancer, Lung Cancer 115 (2018) 116–120.
[29] R.J. Cardnell, L. Li, T. Sen, R. Bara, P. Tong, J. Fujimoto, A.S. Ireland, M.R. Guthrie, S. Bheddah, U. Banerjee, N.N. Kalu, Y.H. Fan, S.J. Dylla, F.M. Johnson, I.I. Wistuba, T.G. Oliver, J.V. Heymach, B.S. Glisson, J. Wang, L.A. Byers, Protein expression of TTF1 and cMYC define distinct molecular subgroups of small cell lung cancer with unique vulnerabilities to aurora kinase inhibition, DLL3 targeting, and other targeted therapies, Oncotarget 8 (43) (2017) 73419–73432.
[30] C.M. Rudin, J.T. Poirier, L.A. Byers, C. Dive, A. Dowlati, J. George, J.V. Heymach, J.E. Johnson, J.M. Lehman, D. MacPherson, P.P. Massion, J.D. Minna, T.G. Oliver, V. Quaranta, J. Sage, R.K. Thomas, C.R. Vakoc, A.F. Gazdar, Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data, Nat. Rev. Cancer 19 (5) (2019) 289–297.
[31] D. Carbone, D. Morgensztern, S.L. Moulec, R. Santana-Davila, N. Ready, C. Hann, B. Glisson, A. Dowlati, C. Rudin, S. Lally, S. Yalamanchili, J. Wolf, R. Govindan, B. Besse, Efficacy and safety of rovalpituzumab tesirine in patients with DLL3-expressing, ≥3rd line small cell lung cancer: results from the phase 2 TRINITY study, ASCO Annual Meeting; Abstract #8507, (2018).
[32] A.C. Tiberghien, J.N. Levy, L.A. Masterson, N.V. Patel, L.R. Adams, S. Corbett, D.G. Williams, J.A. Hartley, P.W. Howard, Design and synthesis of Tesirine, a clinical antibody-drug conjugate pyrrolobenzodiazepine dimer payload, ACS Med. Chem. Lett. 7 (11) (2016) 983–987.
[33] D. Morgensztern, B. Besse, L. Greillier, R. Santana-Davila, N. Ready, C.L. Hann, B.S. Glisson, A.F. Farago, A. Dowlati, C.M. Rudin, S. Le Moulec, S. Lally, S. Yalamanchili, J. Wolf, R. Govindan, D.P. Carbone, Efficacy and safety of Rovalpituzumab Tesirine in third-line and beyond patients with DLL3-Expressing, Relapsed/Refractory small cell lung Cancer: results from the phase II TRINITY study, Clin. Cancer Res. (2019).
[34] Phase 3 Trial of Rova-T as Second-line Therapy for Advanced Small-Cell Lung Cancer (TAHOE Study) Halted [press release], AbbVie, North Chicago, 2018.
[35] AbbVie Discontinues Rovalpituzumab Tesirine (Rova-T) Research and Development Program [press release], Abbvie, North Chicago, 2019.
[36] M. Giffin, K. Cooke, E. Lobenhofer, M. Friedrich, T. Raum, A. Coxon, Targeting DLL3 with AMG 757, a BiTE® antibody construct, and AMG 119, a CAR-T, for the treatment of SCLC, J. Thorac. Oncol. 13 (10) (2018) Supplement.
[37] S. Hipp, V. Voynov, B. Drobits-Handl, F. Trapani, C. Giragossian, J.M. Scheer, P.J. Adam, A Novel T-cell Engaging Bispecific Antibody Induces Specific and Efficacious Lysis of Small Cell Lung Cancer Cells in Vitro and Potent T Cell Redirected Anti-tumor Activity in Vivo (Abstract #549), AACR, Atlanta, (2019).