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Original article

Evaluation of DLL3 Expression in Small Cell Lung Carcinoma in Southern Brazil and its Correlation with EGFR, PDL-1, CICLIN D 1, Neuroendocrine Markers and Clinical Findings

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Abstract

Small cell lung carcinoma (SCSL) is a rare malignancy whose treatment is palliative. The knowledge of its biology is important for the development of new therapies. The expression of delta like 3 protein (DLL3) is involved in the regulation tumorigenic in SCSL.

The aim of this study was to evaluate the expression of DLL3 in small cells lung carcinoma (SCLC) and its correlation with clinical data, survival and association with other biomarkers.

Methods: a cohort retrospective of 56 patients from institution in Southern Brazil was analyzed. The expression of DLL3 was positive when present in 5% or more of the membranes and cytoplasm of neoplastic cells. PDL -1 and EGFR were positive when expressed in 1% or more of the membranes, ciclin D1 and KI 67 by the percentage of stained nucleus.

Synaptophysin, chromogranin and CD56 were tabulated with 1 positive and zero for negative. DLL 3 expression was evaluated as mean, standard deviation and quartiles. Clinical-demographic and death data were analyzed using Fisher's exact test and Pearson's test. Cox regression and the Kaplan-Meier curve were used for survival.

Results: Of 56 individuals, 16 were excluded because there was no tumor available and 13 patients (32.5%) had positive DLL 3.

EGFR expression was 46.2% (HR 2.4), PDL-1 30.8% (HR 3.56) cyclin D1 53.8% (HR 2.77) and chromogranin A 30.8% (HR 0.3)

All patients positive for chromogranin A were negative for anti-DLL3 (p> 0.17). The overall survival for positive DLL3 was slightly higher (p = 0.711) as well as for chromogranin A negative (p 0.299)

Conclusion: The DLL3 mutation acts on SCLC tumorigenesis. The study of its expression may be useful for the development of new therapies. The inverse correlation between DLL3 and chromogranin .A may represented a protective factor, but it needs to be better studied in a larger cohort.

Keywords: ***Small-cell lung carcinoma; DLL3 protein; immunological checkpoints; biomarkers; immunohistochemistry; target drugs.***

Introduction

Neuroendocrine small-cell lung carcinoma (SCLC) is a rare and extremely aggressive malignancy closely associated with the intensity and duration of smoking [1]. It has an aggressive clinical course and is resistant to chemotherapy and standard radiotherapy [1,2].

The American Cancer Society's estimates for lung cancer in the United States for 2020 are about 228,820 new cases of lung cancer (116,300 in men and 112,520 in women) and around 135,720 deaths from lung cancer (72,500 in men and 63,220 in women) [3,4].

In Brazil for the triennium (2020-2022) the estimates for lung cancer is 17760 new cases for men, occupying the third place and 12440 new cases for women, occupying the fourth place of all cancer. In the South region of Brazil the number of cases estimated is 31.07% for man and 12.09% for women [4,5].

The SCSL accounts for approximately 10 to 15% of lung cancer cases and the mortality rate in large series have an estimate of 10% with the relative average of 5-year survival of 15.7% [4,5]. So far, there are no effective therapeutic options. Treatment is palliative with high recurrence rates. The identification of Delta-like protein (DLL3) expression by neoplastic cells in SCLC and the knowledge of immunological checkpoints enabled the production of new target drugs like the antibody-drug conjugate, rovalpituzumab. This drug was being tested in a randomized study on SCLC patients and that was interrupted in phase III due to the cytotoxic effects [6,7].

The DLL3 biomarker is a ligand that inhibits the Notch signaling pathway essential for neuroendocrine development, involved in the regulation of the tumorigenic and regulated directly by Achaete-scute complex-like1(ASCL-1-family of transcription factors) [8,9,10,11,12].

Other immunohistochemical markers are expressed by SCLC cells to a lesser extent, such as the receptor tyrosine kinase (EGFR), present in 16% and the programmed death receptor PD-L1 in about 1.8% of the cases [13,14,15,16,17].

Recently, the Food and Drug Administration (FDA) approved immunotherapy drugs for advanced SCLC stages, anti-PD-L1 (atezulizumab), anti-PD-1 (nivolumab), and nivolumab plus ipilimumab- study CheckMate-032 study) [18,19,20,21,22].

The objective of our study was to characterize the immunohistochemical expression of DLL3 in SCLC in a referral hospital in the south region of Brazil and evaluate its association with clinic pathologic features, survival and its correlation with the antibodies anti-EGFR, anti-PD-L1, cyclin D1 and the panel biomarkers used in routine [23].

Material and Methods

A retrospective cohort of 56 samples from patients diagnosed with SCSL, fixed in 10% buffered formalin and embedded in paraffin was selected in the archives of the pathological anatomy and mycology laboratory of the Irmandade Santa Casa de Misericórdia de Porto Alegre, Brazil (ISCMPA), betwen 2006 at 2016.

Of the 56 patients with SCSL sixteen patients were excluded due to the scarcity of embedded material for immunohistochemical examinations. This retrospective study was conducted according to the ethical standards of the Helsinki Declaration and was approved by the Irmandade Santa Casa de Misericórdia de Porto Alegre ethics committee. The requirement of informed consent was waived because this research did not involve any risk to the participants; the waiver will not adversely affect the rights and well-being of the participants, scant samples have been preserved and data confidentiality maintained.

DLL3 expression on neoplastic cells and clinical findings

Specimens fixed in formalin and embedded in paraffin were used to prepare the slides. To evaluate the immunohistochemical expression of anti-DLL3 (100059 clone), anti-PDL-1 (Cal-10 Clone), anti-EGFR (SP111 clone) and anti-cyclin-D1 (SP4-R clone) antibodies were achieved sections of 2-microns, mounted on silanized slides and submitted to automated immunohistochemical processing.

The expression of membrane and/or cytoplasmic at any intensity was considered positive for DLL3, when present in 5% or more in of neoplasic cells. (**Figures 1,2**).

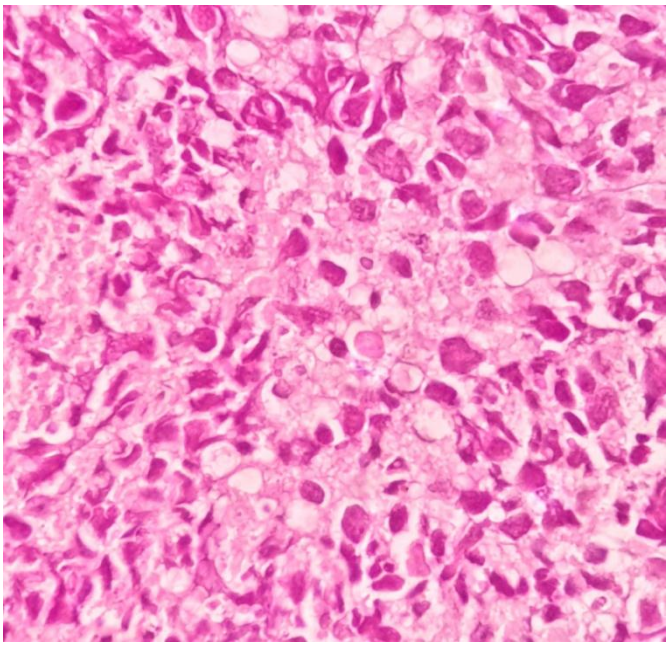


Figure1: Photomicrography.100x increase:H&E-Carcinoma of small lung cells

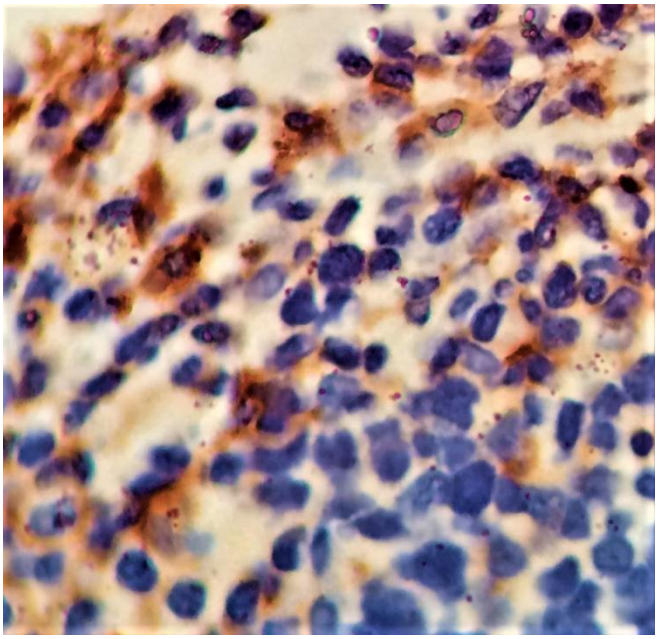


Figure 2: Photomicrography with an increase of 100x. Immunohistochemistry. Anti-DDl3.Carcinoma of small lung cells. Presence of strong cytoplasmic of neoplastic cells

The expression of PDL-1 and EGFR were evaluated by percentage of membrane stained in 1% or more of neoplasic cells.

Biomarkers previously analyzed in routine examinations were pooled in a table and assessed in a binary form: 1 for positive and 0 for negative, including: CK7, TTF1, Synaptophysin, Chromogranin, and CD56. Clinical data were collected from patient records and tabulated. (See the Methods section in the Supplementary Appendix)

Statistical Analysis

The statistical analysis for biomarkers expression were described as a function of mean, standard deviation, frequencies, and quartiles. Comparisons between quantitative data were formed by the Mann-Whitney U test and categorical data by the Fischer exact test. The Kaplan-Meier curve with statistical significance by the log rank test were used for the association with survival. Survival was defined as a period between the date of diagnosis and the date of death or last record. Association strength based on the hazard ratio (HR) and their respective confidence intervals (CIs) were estimated using Cox proportional hazards regression.

Data were stored in the SPSS software for Windows (version 22.0), and p values <0.05 were considered significant.

This study was approved by the Research Ethics Committee of the Irmandade Santa Casa de Misericórdia of Porto Alegre under opinion No. 1,856,228, of December 8, 2016. (See Supplementary Appendix)

Results

Patient Characteristics

Clinical data are summarized in table 1. The mean age of patients who were positive for DLL3 was 65.3-or+10.1, ranging from 42 to 89 years old. The presence of metastasis was 76.9% and smoking, 76.9% for patient DLL3 Positive.

Table 1: Clinical characteristics of patients with SCLC and their correlations with positive DLL3 vs. negative DLL3

Characteristics	DLL3 (+) n = 13	DLL3 (–) n = 27	P value
Age, years	65.3±10.1	67.7±10.5	0.480
Female sex, number (%)	6 (46.2)	12 (44.4)	>0.99
Metastasis, number (%)	10 (76.9)	21 (77.8)	>0.99
Smoking, number (%)	10 (76.9)	23 (85.2)	0.662
SAH, number (%)	7 (53.8)	13 (48.1)	>0.99
Dyslipidemia, number (%)	0 (0.0)	2 (7.4)	>0.99

DLL-3 Expression and correlation with biomarkers

Of the 40 patients suffering from SCLC included in the study to assess the expression of the DLL3 biomarker, 13 (32.5%) showed membrane and/or cytoplasmic expression in 5% or more of neoplastic cells, being 7 (53.8%) men and 6 (46.2%) women.

In positive DLL3 patients, 46.2% had membrane expression equal or more than 1% for the EGFR biomarker (OR: 2.04 and 95% CI: 052-8.00); 30.8% for PDL-1 (OR: 3.56 and 95% CI: 0.66-19.11) and 53.8% Cyclin D1 (OR: 2.77 and 95% CI (0.71-10) Table 2.(See Supplementary Appendix)

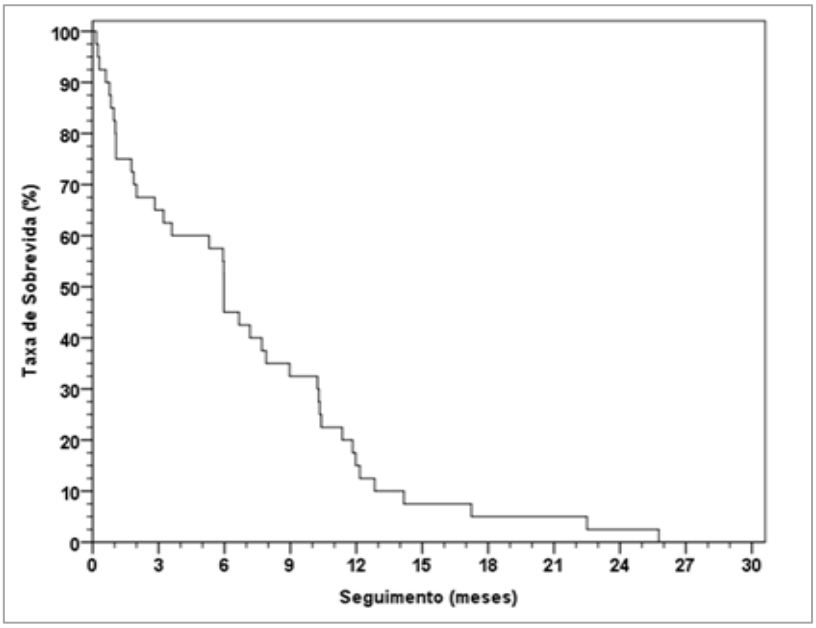
By assessing the markers previously examined in the laboratory routine, we observed a tendency of chromogranin to show less nuclear expression in DLL3 positive patients (30.8%) in comparison with DLL3 negative patients (60%), representing an inverse correlation, which probably could act as a protective factor (OR: 0.3 and 95%CI: 0.07-1.23) (Table 2).

Table 2: Distribution of positive results for selected markers among SCLC patients with positive DLL3 versus negative DLL3

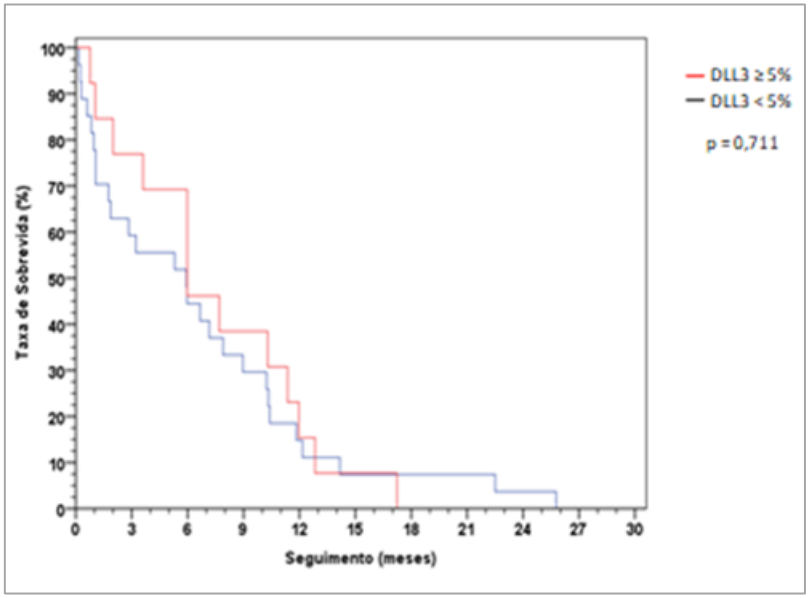
Positive markers	DLL3 (+) n = 13	DLL3 (–) n = 27	OR	95%CI	P value
Chromogranin A, number/ not evaluated (%)	4.13 (30.8)	15/25 (60.0)	0.30	0.07 - 1.23	0.170
CK7, number (%)	11 (84.6)	20 (74.1)	1.93	0.34 - 10.92	0.690
Synaptophysin, number/ not evaluated (%)	9/12 (75.0)	23/26 (88.5)	0.39	0.07 - 2.31	0.357
CD56, number (%)	13 (100.0)	23 (85.2)	5.17	0.25 - 104	0.284
TTF1, number (%)	12 (92.3)	25 (92.6)	0.96	0.08 - 11.66	>0.999
EGFR, number (%)	6 (46.2)	8 (29.6)	2.04	0.52 - 8.00	0.480
PD-L1, number (%)	4 (30.8)	3 (11.1)	3.56	0.66 - 19.11	0.187
Cyclin-D1, number (%)	7 (53.8)	8 (29.6)	2.77	0.71 - 10.88	0.175
Ki-67, number (%)	13 (100.0)	23 (85.2)	5.17	0.25 - 104	0.284

Outcome Survival

Overall survival for SCLC patients was six months. Median survival was 5.97 (Figure 3).



In figure 4 the survival rate is evaluated in patients suffering from SCLC who were positive (> or equal to 5%) and negative for DLL3 (<5%) (Figure 4). The Kaplan-Meier curve shows that DLL3 positive patients have a slightly higher survival rate (p 0.711).



The survival rate in positive chromogranin patients using the Kaplan-Meier test was slightly higher (p 0,299).Figure 5

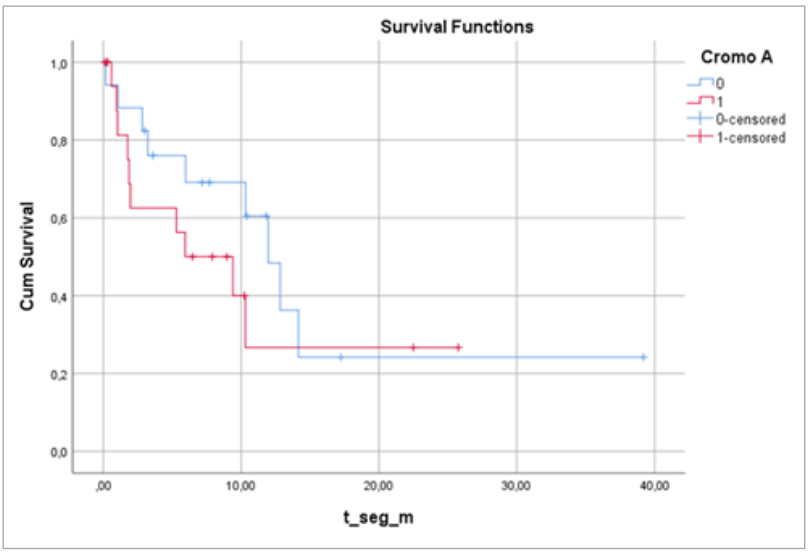


Figure 5: survival as a function of the association between DLL3 and chromogranin A

Discussion

SCLC is a low-incidence neoplasm whose treatment is limited to chemotherapy, radiotherapy and, more recently, immunotherapy in advanced stages. For the development of new systemic strategies it is necessary to understand oncogenesis in SCLC [22,23].

Saunders et al., in 2015, in a preclinical study in vitro and with xenograft in mice demonstrated the expression of DLL3 by SCLC neoplastic cells and concluded that this ligand from the Notch pathway acts as a predictive factor of response to treatment [24].

In 2018, Kentaro et al., reported in a cohort with 63 patients an expression of DLL3 of 83%. In that study there was no correlation between the expression of DLL3 and clinical, demographic or survival [25].

More recently, in 2019, Furata et al., in a cohort of 93 SCLC patients undergoing surgical resection, detected 83% of DLL3 expression. There was no statistical correlation between DLL3 and patient survival. However, the authors described that DLL3 acts as a regulator of cell migration and invasion in SCLC, suggesting that it plays a role as an oncogene in neuroendocrine neoplasms [26].

In addition, in July 2019, Owen et al., evaluated the expression of DLL3 by immunohistochemistry in 63 samples from patients with SCLC, of which 52 (83%) were positive. This study addresses the possibility of developing new therapies for SCLC such as antibody-drug conjugate, bispecific immunological therapy with involvement of T cells and chimeric antigen receptor, which redirects CD3 positive T cells to positive DLL3 tumors and promotes cell apoptosis. Near-infrared photoimmunotherapy (NIR-PIT) has also been studied as strategy aimed at DLL3 for the treatment of SCLC [27].

In our study, the expression of DLL3 in neoplastic cells was 32.5%. As Kentaro et al. (2018), there was no correlation with clinical-demographic findings. We assessed the correlation between positive DLL3 and EGFR, PDL-1, Cyclin D1 and CD56. The HR values (table 2) indicate clinical impact on this correlation, although without statistical confirmation.

However, the finding that came closest to the stipulated statistical value was the correlation between DLL3 and chromogranin A. Positive DLL3 patients showed low expression of the biomarker chromogranin A (OR = 0.30 and IC-007-1.23), raising the hypothesis that there as inverse correlation between the two biomarkers. Thus, this correlation would represent a greater tendency for protective action.

In the survival assessment, the positive DLL3 patient survives a little longer. However, around the 15th month, mortality in both groups is the same. In groups of positive DLL3 patients with negative chromogranin, the survival rate by the Kaplan-Meier test was slightly higher (p 0.299) [28,29].

Our data on the expression of chromogranin A corroborate with the studies by Kowalki et al. The authors report that chromogranin and synaptophysin are associated with SCLC survival and that they act as an indirect predictive factor. Neoplasms with high rates of chromogranin contain a greater amount of secretory vesicles in neuroendocrine cells, conferring a strong potential for malignant transformation [28,29].

Our study, carried out in southern Brazil, had several limitations. First, SCLC is a rare disease and we retrospectively assessed a small cohort of patients. In addition, differences in antibody choice, scoring method and cut-off values can impact immunohistochemical assessment and results. Considering the small size of the group of patients with positive DLL3, a multivariate analysis may not adequately adjust the association of DLL3 expression with clinical results. On the other hand, we have a great inherent challenge, represented by the heterogeneous tumor microenvironment, in which incisional biopsies may not be representative of a specific genetic alteration.

We found strong evidence in the literature to consider that DLL3, PDL-1 and EGFR are interdependent markers, reinforcing the Delta-Like 3 study: the Notch3 and EGFR / MAPK pathways cooperate in modulating apoptosis;[31].

Notch-1 expression is positively regulated by EGFR-TKIs, promoting neoplastic cell resistance to therapies; [32] the activation of (EGFR) by its binding to the ligand (EGF) induces the expression of PD-L1 in NSCLC cells, through PI3K / Akt / mTOR [33]; preclinical data show that Rova-T increases the expression of PDL1 in tumor cells and the infiltration of immune cells in the tumor, suggesting that Rova-T may change the tumors to a more responsive state at the checkpoint [34].

In conclusion, we emphasize that the Notch pathway, when mutated, activates the DLL3 protein and alters the ASCL1 transcription, acting on CPCP tumorigenesis. Based on this knowledge, research involving the Notch pathway and its interdependence with other pathways in the cell cycle may be beneficial for the development of new target therapies

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Conflict of interests:

The authors declare that there is no conflict of interest

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