Diplomarbeit

Analysis of immunohistochemical expression of DLL3, TP53 and RB1 in the patients with small cell and large cell neuroendocrine carcinoma of the lung

eingereicht von

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Graz, 3. Januar 2019

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 03.01.2019 *Christian Kuchler eh*

Declaration

The following results have also been presented as a part of a manuscript which is currently submitted for publication in a peer-reviewed journal:

"Comparison of four DLL3 antibodies performance in high grade neuroendocrine lung tumor samples and cell cultures" by Luka Brcic, Christian Kuchler, Sylvia Eidenhammer, Daniela Pabst, Franz Quehenberger, Adi Gazdar, and Helmut Popper.

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Zusammenfassung

Einleitung: Lungenkrebs ist auch heute noch einer der häufigsten bösartigen Erkrankungen in der westlichen Welt. Diese bösartigen Lungentumore sind für fast 1/5 aller Krebs-assoziierten-Todesfälle verantwortlich. Insbesondere Patientinnen und Patienten mit kleinzelligen Lungenkarzinomen werden meist erst in einem fortgeschrittenen Stadium der Krankheit diagnostiziert und haben oft eine schlechte Prognose. Dies beruht meist auf der frühen Metastasierung, dem schnellen Tumorwachstum und der zentralen Lokalisierung des Tumors.
Darüber hinaus, gab es bei der Therapie dieser Erkrankung über die letzten Jahrzehnte fast keine Fortschritte. Jüngst konnte jedoch ein neuer Hoffnungsschimmer für die Patientinnen und Patienten geweckt werden. Eine klinische Phase-1 Studie, die ein Antikörper-Wirkstoff-Konjugat gegen DLL3 (Rovalpituzumab tesirine) testet.
Um diese neue Form der Therapie anzuwenden, braucht es jedoch einen immunhistochemischen Nachweis der DLL3 Expression im Tumorgewebe.
Diese Testung wird dadurch erschwert, dass in der klinischen Studie ein Antikörper verwendet wurde, der nicht in jedem pathologischen Labor verfügbar ist. In dieser Studie wurden 4 unterschiedliche Antikörper verwendet um die Expression von DLL3 in kleinzelligen Lungenkarzinomen (SCLC) und großzelligen neuroendokrinen Karzinomen (LCNEC) nachzuweisen.

Methoden: Die Studiengruppe setzt sich aus 53 chirurgisch entfernten Proben zusammen. 29 davon waren LCNEC, 24 SCLC. Von jedem einzelnen Fall wurden Tissue micro arrays (TMA) angefertigt und danach unter Verwendung der verschiedenen Antikörper immunhistochemisch analysiert. Die entstandenen Bilder der Färbung wurden hinsichtlich der Expression von DLL3 mikroskopisch analysiert und manuell ausgewertet. Der Antikörper, der in der klinischen Studie verwendet wurde, bildete die Referenz.
Darüber hinaus wurde eine Validierungsgruppe mit 46 SCLC Fällen angelegt, die durch die gleichen Methoden verifiziert und ausgewertet wurde.

Ergebnisse: Die Expression von DLL3 war, unabhängig vom Typ des Tumors und den Cut-Off Werten, mit jedem verwendeten Antikörper unterschiedlich. Der Referenzantikörper der klinischen Studie zeigte gegenüber den drei anderen Antikörpern eine Überlegenheit. Er war richtig-negativ bei Zellen, die DLL3 nicht exprimiert hatten

und richtig-positiv, bei Zellen mit einer nachgewiesenen DLL3 Expression. Darüber hinaus konnte keine Korrelation zwischen der Expression von DLL3 und einem Verlust des RB1 in LCNEC und SCLC beobachtet werden.

Abstract

Introduction: Lung cancer is one of the most prevalent malignancies in the World. Nearly one out of five cancer related deaths is caused by lung cancer. Patients with small cell lung cancer are diagnosed at an advanced stage and have a very poor prognosis due to early metastasis and rapid growth. Unfortunately, until today there is no significant improvement in therapy. Recently, a clinical phase 1 study of an antibody-drug conjugate against DLL3 (Rovalpituzumab tesirine) showed promising results. In order to identify patients with a higher probability of response to this therapy, DLL3 expression analysis is required. However, not all pathological laboratories will be able to use antibody applied in clinical study, due to the need of adequate technical equipment. Because of this, we aimed to compare concordance of expression of four different DLL3 antibodies in LCNEC and SCLC.

Methods: Study cohort included 53 surgically removed samples of LCNEC (29) and SCLC (24). Tissue microarrays were constructed and used for immunohistochemical analysis. Four different antibodies targeting DLL3 were used. Slides were analysed under microsope. Percentage of positive tumour cells, and H score (percentage of positive tumour cells multiplied by intensity of the staining) were recorded. Standard antibodies for p53 and Rb1 were also used. Staining results for both were expressed as percentage of positive tumour cells. The same staining was repeated in validation cohort, consisting of 46 SCLC. Obtained results were correlated with survival data.

Results: The expression of DLL3 was different with each antibody, regardless of the type of tumour and the used cut-off values. Furthermore, no correlation between the expression of DLL3 and a RB1 loss was observed in LCNEC. In SCLC we have found a positive correlation of DLL3 expression with a loss of RB1.

Conclusion: We have shown that results obtained using different DLL3 antibodies are not concordant, and these antibodies are not interchangeable. Evaluation of DLL3 expression, is however, feasible, and it was present both in SCLC and in LCNEC, but without any prognostic significance.

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List of Abbreviations

ALK	Anaplastic lymphoma kinase
ASCL1	Achaete-scute-homolog 1
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CK5	Cytokeratin 5
COPD	Chronic obstructive pulmonary disease
CT	Computer tomography
EGFR	Epidermal growth factor receptor
FGFR1	Fibroblast growth factor receptor 1
FHIT	Fragile histidine triad
HER-2	Human epidermal growth factor receptor 2
HPV	Human papillomavirus
LCNEC	Large cell neuroendocrine carcinoma
LKB1	Liver kinase B1
MDM2	Mouse double minute 2 homolog
MEN1	Multiple endocrine neoplasia type 1
MRI	Magnetic resonance imaging
NCAM	Neural cell adhesion molecule
PD-L1	Programmed cell death 1 ligand 1
PET-CT	Positron emission tomography – computed tomography
RASSF1	Ras association domain-containing protein 1
RB1	Retinoblastoma protein1
SCNEC	Small cell neuroendocrine carcinoma
SCLC	Small cell lung carcinoma
SIADH	Syndrome of inappropriate antidiuretic hormone secretion
SOX2	Sex determining region Y – box 2
TMA	Tissue microarray
TTF1	Thyroid transcription factor 1
VHL	von Hippel-Lindau tumor supressor

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1 Introduction and Background

1.1 Epidemiology

Lung cancer is still one of the most prevalent forms of cancer in the modern society and remains at the top of the cancer statistics in men (1). In women, mostly due to the different amount of tobacco smoked, lung cancer remains at the second place, right after breast cancer, which is the most common cancer among the female population (2). In 2018 alone, there were 2.1 million new cases recorded worldwide, with almost 90% appearing in high and very high developed countries. The highest incidence rates in the male population, as seen below (Figure 1), are found in Micronesia and Polynesia (54.1 and 52.0 per 100,000) the central and eastern part of Europe (49.3 per 100,000) and the eastern part of Asia (47.2 per 100,000). Countries in the western part of Africa as well as Eastern Africa are listed at the bottom of the statistics (2.4 and 3.4 per 100,000) (1).

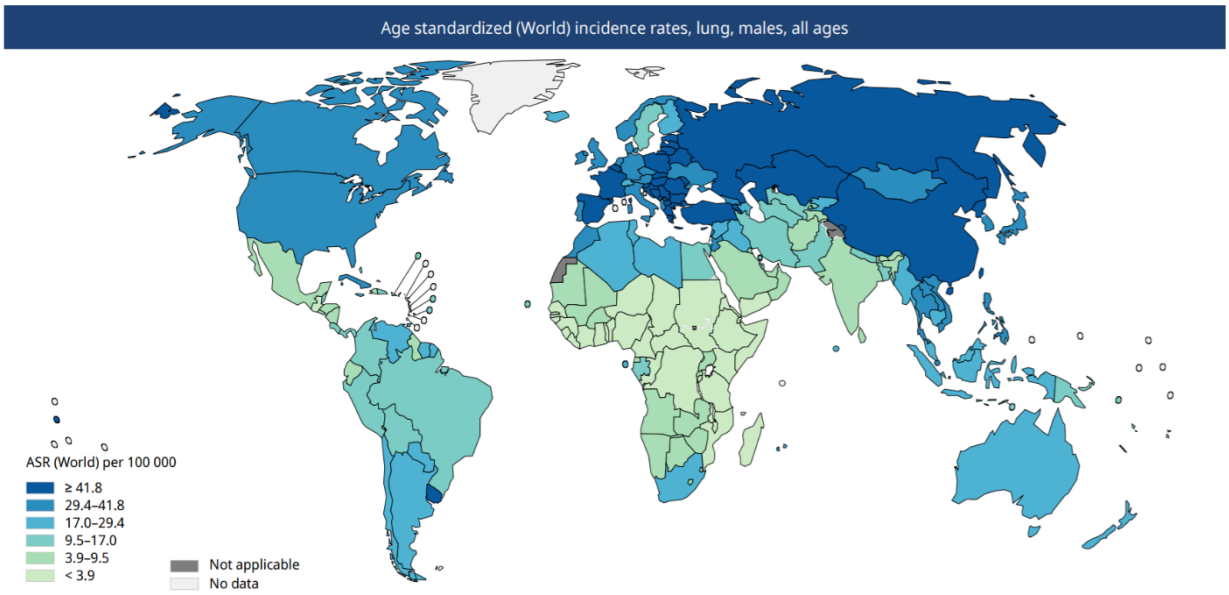

Figure 1: Estimated Lung Cancer Incidence Worldwide in 2018: Men (1)

However, if we compare this to the female population (Figure 2), the incidence slightly changes in geography, with the highest number of patients found in North America (30.7 per 100,000) and the northern part of the European continent (26.9 per 100,000), followed by Western Europe (25.7 per 100,000). Very low rates are found in the African countries (1).

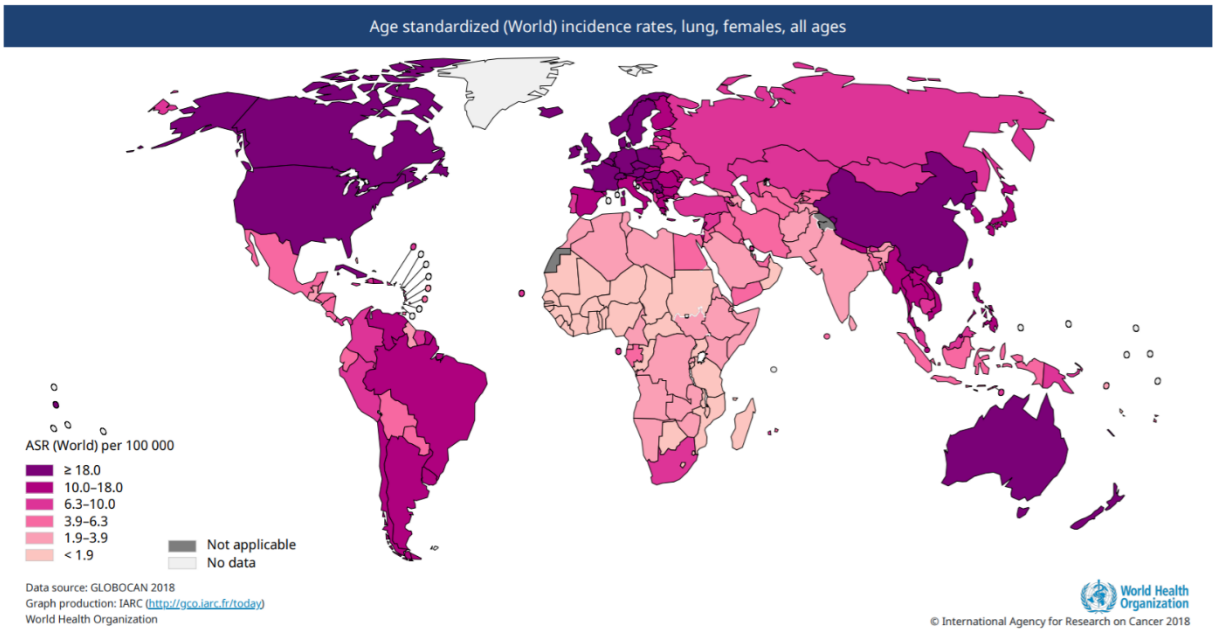

Figure 2: Estimated Lung Cancer Incidence Worldwide in 2018: Women (1)

Looking at the mortality of cancer worldwide, within both sexes, lung cancer is still the most frequent cause of death. Almost one out of five cancer related deaths is due to malignant lung tumour (1.76 million per year) (1).

The situation in Austria is slightly different. In 2015, lung carcinoma was diagnosed in 2.956 male and 1.904 female patients. In the same year 3.889 people (2.396 male/1.493 female) died and 13.242 patients (7.589 male/ 5.653 female) lived with lung cancer. These numbers make lung cancer the second most common cancer in women and the second most frequent in men in Austria. Lung cancer is the leading cancer related death in men in Austria, and at the second place in women. The female lung cancer incidence and mortality increased significantly (+33% and +30%, respectively), while the male rates showed decrease (-10% and -17%, respectively). Geographically, Upper Austria has the lowest mortality, as well as the lowest incidence, while Vienna has the highest rates in both categories in the period between 2013 and 2015 (3).

The relative one-year survival between 1998-2002 and 2013-2015 improved moderately (from 40% to 50%), while five-year survival rate increased by 4% (from 15% to 19%). Generally, men in Austria have a lower probability of surviving five years with lung cancer than women (3).

1.2 Etiology

1.2.1 General risk factors

Carcinogenesis of a malignant lung tumour is multifactorial. However, nearly 90% of all malignancies in the lung are triggered through smoking cigarettes, both active and passive. Other pulmonary carcinogens, like radioactive radiation, radon, arsenic or asbestos, have a smaller significance in the carcinogenesis of these tumours. Inhaled fine dust could also play a role in lung carcinogenesis, but that is still under investigation. It is important to stress that lung cancer develops also in non-smokers , but in many cases driven by targetable mutations. (4).

1.2.2 Smoking exposure

Over 7000 different chemicals are hidden within tobacco smoke, of which 60 of them were proven as carcinogens in animal experiments. More important, it does not make a difference if a person uses filter or non-filter cigarettes, e-cigarettes or light cigarettes. All of them contain toxins like polycyclic aromatic hydrocarbons, aldehydes, heavy metals or aromatic amines, which can all cause cancer (4).

The fact that the incidence of lung carcinoma in non-smokers (3,4/100.000/year) is about 15 times lower than in smokers who smoke 10 cigarettes a day (51,5/100.000/year), and about 63 times lower than in heavy smokers who smoke 40 cigarettes a day (217,5/100.000/year), illustrates that smoking is the main trigger in the carcinogenesis of lung cancer (4). Smokers make up by far the biggest proportion of people diagnosed with lung cancer worldwide (5).

The longer someone smokes and the more cigarettes someone consumes daily, the higher is the risk of developing a malignant lung tumour. Moreover, after smoking cessation the risk drops continuously. (6). Therefore, smoking cessation is a crucial part of the prevention and therapy of lung cancer (7).

1.3 Clinic

1.3.1 Symptoms

Typical first alarm signs and symptoms of lung cancer are cough, huskiness, haemoptysis, chest pain, stridor and dyspnoea. However, some patients are symptomless and don´t have any problems. Nevertheless, the further the disease spreads, the more symptoms occur. Due to metastatic processes, people start to lose weight, have pain in the abdomen or spinal column due to liver or bone metastases or neurological symptoms due to brain metastases.(8). During the progression of the disease, more than 20% of the people diagnosed with a malignant lung tumour generate metastases in the brain or spinal cord, which can lead to nerve entrapment or epileptic seizures (9).

1.3.2 Imaging

Thoracic X-ray is still the most common imaging, which is performed on suspicion of a malignant lung tumour. The lung carcinoma can present as a pleural infusion, atelectasis or a round mass. However, a positive result in thoracic X-ray, as well as a negative result, that does not agree with the clinic, leads to the necessity of a more detailed imaging such as thoracic and epigastric CT with contrast agent (signs of metastasis, infiltration or contrast agent enhancement). Morphologically, lung carcinomas often show typical signs such as pleural retraction, necrotic areas and vascular signs. Although the CT has a high sensitivity, the specificity is rather poor. Therefore, especially for the TNM staging, an MRI is preferentially used. To confirm the radiological diagnosis a transbronchial or transthoracic biopsy, depending on the location of the tumour, is used.
Further staging often requires a PET-CT, bone scintigraphy or sonography.
According to the new guidelines, imaging plays an important role in the screening of lung cancer. Patients between the age of 55 and 74 who are under risk (more than 30 pack years and less than 15 years without smoking) or patients over the age of 50 with more than 20 pack years and an additional risk factor (COPD, lung fibrosis, family members with lung cancer etc.) should get an annual low-dose CT (10).

1.3.3 Paraneoplastic symptoms

Lung cancer is one of the tumours with the highest occurrence of paraneoplastic symptoms. However, different subtypes of the malignant lung tumours have different probabilities of developing such symptoms. In adenocarcinomas paraneoplasia is found very rarely, while small cell lung carcinomas tend to produce neuropeptides with the effect of pituitary hormones (11) (12). This can lead to hyponatremia (through SIADH) hypercalcaemia (through parathormone-like substance), gynaecomastia (through gonadotropins), increased pigmentation (through melanocyte-stimulating hormone), Cushing syndrome (through adrenocorticotropic hormone) or prolactinaemia (13,14). Neurological deficits, such as encephalopathy, myasthenic syndromes, autoimmune neuropathies or Lambert-Eaton myasthenic syndrome can also occur in the context of a paraneoplastic syndrome (8). The blood system, bone marrow and immune system can also be affected. Anaemia, thrombocytopenia, a nephrotic syndrome, the systemic lupus erythematosus or a disseminated intravascular coagulation, to name just a few, can be the consequence (13).

1.4 Histological Subtypes

According to the WHO-Classification, there are more than 50 histologically different lung carcinomas (4). Generally, there are two main histological groups. The small cell lung carcinomas (SCLC) (15% of the cases) and the non-small cell lung carcinomas (NSCLC) (85% of the cases) (15). The most frequent histological type is adenocarcinoma, followed by squamous cell carcinoma and then small cell carcinoma. Rather rare are adenosquamous carcinomas, pleomorphic carcinomas, spindle and giant cell carcinomas, followed by many different rare tumours (4). If all possible differentiations/classifications are excluded, morphologically and immunohistochemically, tumours are classified as a large cell lung carcinoma.

1.4.1 Adenocarcinoma

The adenocarcinoma makes up the largest part of the malignant lung tumours. Today, more than 40% of all lung carcinomas worldwide are adenocarcinomas, and the number of newly diagnosed adenocarcinomas is still rising.
The number has increased considerably since the 1980s. At that time, less than 30% of all lung malignancies were adenocarcinomas. At the same time squamous cell carcinoma numbers reduced from 32% to 20%, while incidence of small cell carcinoma remained practically the same (17% to 13%), and large cell neuroendocrine carcinoma also showed decrease in incidence (8% to 2%) (8).

The main cause of this change is the “improvement” in cigarette production. The companies added ventilation holes and filters, therefore reducing the amount of tar. Consequently, the nicotine content was reduced too. That made the smokers inhale deeper to enhance the puff volume. The result was an increased peripheral load of the harmful substances and a rise in the incidence of adenocarcinomas.
Another important reason why the adenocarcinomas are more frequent nowadays is the fact, that the diagnostics made tremendous progress within the last few years. Especially development and improvement of immunohistochemistry, leading to application of adenocarcinoma and squamous cell carcinoma markers in everyday diagnostic procedures. (8) (16).
Lung carcinoma metastases occur relatively frequently. They first spread into the regional ipsilateral as well as contralateral lymph nodes (hilar and mediastinal), and then through the blood into adrenal glands, brain, bones and the lung itself. In addition, liver metastases

are very common in this type of carcinoma too. Moreover, the metastasis can also involve the pleura, which is associated with a poor survival (17).

Histologically adenocarcinoma shows different growth patterns; solid, micropapillary, papillary, acinar and lepidic. They might also produce mucin, and according to the latest WHO classification there are some special variants like colloid, fetal and enteric adenocarcinoma.(8).

Today, the most important immunohistochemical marker of adenocarcinoma is TTF1. It is expressed in nearly 75% of the lung adenocarcinoma cases and shows a correlation with the EGFR mutation (18) (19) (20).
This marker is almost always expressed in lepidic and papillary adenocarcinomas, whereas it can be negative in solid subtypes (21). However, TTF1 is also found in a variety of other tumours, like neuroendocrine tumours of the lung and thyroid malignancies (8).

Lung adenocarcinoma harbours a large variety of mutations, some of which are "druggable" and therefore improve survival (8). Of the highest clinical importance are activating mutations in the EGFR, ALK and ROS-1 gene, as well as in BRAF and HER2, since they are all targetable mutations with available therapy. (22) (23) (24) (25). Another aberration commonly found in adenocarcinomas is a mutation in the Kirsten rat sarcoma viral oncogene (KRAS) (26)(27)(28). However, although it is very common (ca 25-30%), there are still no KRAS targeted therapies today.

Overall, women and never-smokers have better prognosis, regardless of the clinical stage. Patients diagnosed with a carcinoma bigger than 2.5cm, or demonstrating micropapillary or predominant solid growth pattern are associated with a poorer survival (29) (30) (31) (28) (32).

1.4.2 Squamous cell carcinoma

The squamous cell carcinoma was once, with 32% of all diagnosed cases, the most common lung cancer. However, due to a previously explained changes in the smoking behaviour and the cigarette production, it is now the second most frequent after the adenocarcinoma (8).

People who start smoking in their teenage years or consume tobacco products over a very long period of time have a significantly increased risk of developing the squamous cell lung carcinoma. Furthermore, the amount of tobacco which a person consumes every day is related to the incidence of developing squamous cell lung carcinoma (33).
A further well documented risk factor is the exposition to arsenic (34) (35) (36), while some controversial studies claim that there is a connection between this type of cancer and a HPV infection (37) (38).

Generally, this tumour is very aggressive and mainly located near the bronchus. Histologically the typical features of this epithelial tumour, including intercellular connections, “pearl formation” as well as keratinization, appear in the keratinizing subtype and are often lost through a lack of differentiation of the carcinoma. In the non-keratinizing subtype these features are rarely found. Third subtype is basaloid squamous cell carcinoma, characterized by palisading of tumour cells at the borders of tumour cell nests. Immunohistochemistry can help in diagnosis, since p40, p63 or CK5 are here usually positive, while TTF1 is negative (39).

The rate of mutations in squamous cell lung carcinoma is very high (40). Most commonly affected are chromosomes 3q (with the TP63 and SOX2 gene), 7p (with the epidermal growth factor receptor), 8p (with the FGFR1) and 9p (with the CDKN2A gene) (41) (42). Moreover, the tumour suppressor genes TP53, RB1 and the NOTCH1 are often also directly modified or damaged (40). However, so far none of these is “druggable” (39).

1.4.3 Large cell carcinoma

The cases of large cell carcinomas have reduced almost by 75% within the last 30 years, due to a progress in the discovery of immunohistochemical markers for cell differentiation. Many of the formerly diagnosed large cell carcinomas are now listed as non-keratinizing squamous cell carcinomas, basaloid squamous cell carcinomas, solid adenocarcinomas or large cell neuroendocrine carcinomas. Therefore, as a differential diagnosis, all of the above are possible (43).

It is mostly found in men and has a strong association with tobacco consumption. In most cases, the tumour is located in the peripheral part of the lung and diagnosed in patients above the age of60 years (43).

Histologically, the tumour cells are large and polygonal, the nucleoli are striking, and the cytoplasm is described as of average quantity. Generally, it does not show any sign of differentiation, morphologically or immunohistochemically (TTF1, napsin A, p40, p63 negative) (43).

In molecular pathological analyses both mutations usually found in adenocarcinomas, as well as in squamous cell carcinomas of the lung, are detected (42) .If “druggable”, they provide a possibility for targeted therapy in this group of patients (43)

1.4.4 Adenosquamous carcinoma

The adenosquamous carcinoma is defined as a combination of the adenocarcinoma and the squamous cell carcinoma, with both tumour subtypes at least comprising 10% of the tumour. The tumour is also highly associated with smoking and makes up between 0.4% and 4% of all diagnosed lung carcinoma cases (44) (45) (46) (47) (48).

It is typically located in the peripheral part of the lung, but there are also carcinomas found near the bronchus (49) (50) (51).

Concerning genetic changes, this tumour also combines mutations of both adeno- and squamous cell carcinomas. Patients who have a high consumption of tobacco products often show a mutation in the KRAS gene, while in never-smokers, and especially female patients, EGFR mutations are the most common (52) (53) (54). Furthermore, mutations in the HER2 (55), ROS1 (56), ALK (57), LKB1 (58), FGFR1 (59) and RET (60) were also detected in this type of tumour.

The prognosis of the adenosquamous carcinoma is poor, with 5-year survival for resectable stage of only 40% (61) (62) (50) (63).

1.4.5 Neuroendocrine tumours

1.4.5.1 Small cell carcinoma

This type of malignant lung tumour makes up about 13% of the lung cancers newly diagnosed every year. It is almost exclusively associated with heavy tobacco smoking (64). Chronical lung diseases, like COPD or asthma, are also assumed to be a trigger or supplementary risk factor for the genesis of this cancer (65) (66). On the other hand, there are some protective factors, like a hormone replacement treatment, which reduces the risk moderately (67).
Histologically, it has small round or spindle shaped, sometimes oval cells, without pronounced nucleoli and with a small amount of cytoplasm. SCLCs are characterized by a high mitotic rate and necrosis. Furthermore, this cancer commonly produces neuroendocrine peptides or markers, which, as previously mentioned, lead to paraneoplastic syndromes (68).

Together with the typical carcinoid (low grade malignancy), the atypical carcinoid (intermediate grade malignancy) and the large cell neuroendocrine carcinoma (high grade malignancy), it belongs to the group of neuroendocrine lung tumours (69) (70) (71).

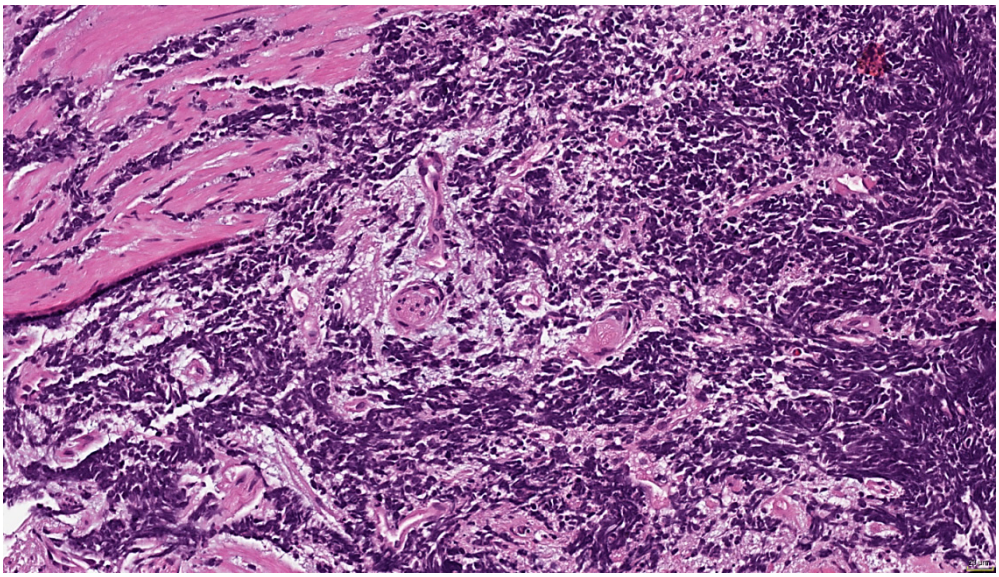

Figure 3: Typical histological presentation of a small cell lung carcinoma.

In contrast to the high-grade malignancies (SCLC, LCNEC), the carcinoids tend to appear in combination with a multiple endocrine neoplasia syndrome type 1 or an ordinary MEN1 mutation (72) (73). They also occur in combination with diffuse idiopathic pulmonary neuroendocrine cell hyperplasia, which is believed to be the precursor lesion for the carcinoids. However, in the high-grade neuroendocrine carcinomas (SCLC, LCNEC) such preinvasive lesion was not found (74).

Rapid growth, early metastasis and paraneoplastic syndromes characterize the SCLC. It is mostly found near the bronchus, but also in the periphery (5%). The poor prognosis is mainly based on the high lymphogenic and haematogenic metastasis rate. The metastases first spread into the regional lymph nodes (intrathoracic and supraclavicular) and then over the blood into liver, adrenal glands, brain, bones and the lung (68). In imaging, such as X-ray or CT, the SCLC presents as a big hilar mass as well as a mediastinal lymphadenopathy or metastasis (75).

The prognosis of SCLC is extremely poor. At a disease stage with metastases the 2-year survival rate is only 10%, while a patient with an early stage SCLC, without metastases, has a chance of 25% to survive the next 5 years (76) (77). On average, a patient diagnosed with SCLC survives only 12.7 months (78). Nevertheless, there are several positive prognostic factors. Younger, female patients, who undergo surgery due to a limited disease stage have a better survival (79) (77). On the other hand, some factors are associated with a shorter survival time. Diagnosed patients who continue smoking, a low level of haemoglobin, metastases in brain, spinal cord or the bones and paraneoplastic syndromes belong to this category (68).

1.4.5.1.1 SCLC Classifications

SCLC are classified using the same TNM system like for NSCLC.

TNM Classification of SCLC (80)

Category	**Stage**	**Description**
T (Tumour)	Tis	Carcinoma in situ
	T1 T1a (mi) T1a T1b T1c	Carcinoma < 3cm, main bronchus not involved, minimal invasive Adenocarcinoma Carcinoma < or = 1cm Carcinoma > 1cm but < or = 2cm Carcinoma > 2cm but < or = 3cm
	T2 T2a T2b	Carcinoma > 3cm but < or = 5cm, infiltration of the main bronchus (not the carina), infiltration of the visceral pleura, tumor related obstructive pneumonia or atelectasis Carcinoma > 3cm but < or = 4cm Carcinoma > 4cm but < or = 5cm
	T3	Carcinoma > 5cm but < or = 7cm, infiltration of the chest wall, infiltration of the parietal pericardium or N. phrenicus Additional: a second tumor in the same pulmonal lobe
	T4	Carcinoma > 7cm or direct infiltration in mediastinum, heart, trachea, esophagus, diaphragma, vertebral body, carina, N.laryngeus recurrens or the big vessels Additional: a second tumor in an ipsilateral pulmonal lobe
N (Lymph node)	N0	No lymph node metastasis

	N1	Lymph node metastasis in ipsilateral hilar/pulmonal lymph node or peribronchial lymph node
	N2	Lymph node metastasis in ipsilateral mediastinal or subcarinal lymph node
	N3	Lymph node metastasis in contralateral mediastinal/hilar lymph node or contralateral/ipsilateral deep cervical or supraclavicular lymph node
M (Metastasis)	M0	No distant metastasis
	M1 M1a M1b M1c	Distant metastasis Additional tumour in a contralateral pulmonal lobe, malignant pleural effusion, malignant pericardial effusion Isolated distant metastasis in an extra thoracic organ > 1 distant metastasis in one or more extra thoracic organs

Table 1: TNM Classification of SCLC - IASLC Lung Cancer Staging Project (80)

Classification of the Veterans Administration Lung Study (81)

1. Very limited disease: T1-2 or/and N0-1
2. Limited disease: T3-4 or/and N2-3
3. Expanded disease: M1 and everything above the limited disease

This classification is based on the feasibility of a radiotherapy. A limited disease stage can be completely covered and irradiated. Although this classification is usually adequate for clinical purposes, more accurate staging systems, such as TNM, are usually used to describe the staging and prognosis of SCLC (81).

1.4.5.2 Large cell neuroendocrine carcinoma

The large cell neuroendocrine carcinoma belongs to the group of neuroendocrine lung tumours. It also produces neuroendocrine peptides and markers, which can be measured immunohistochemically. More than 90% of these carcinomas are found in regular smokers. Moreover, large cell neuroendocrine carcinomas also share the same loss of tumour suppressor proteins and gens, as well as the same carcinogenesis pathway with the small cell carcinoma (82). Furthermore, it is assumed, that the tumour cells of the LCNEC originate from Clara-like cells. These precursors have a neuroendocrine differentiation and were tested in animal models (83) (84).

The LCNEC are mostly found in the peripheral parts of lung. However, in 20% the LCNEC appears near the bronchus. This can cause a bronchial stenosis leading to pneumonia or atelectasis. A paraneoplastic symptom is rarely found in contrast to the small cell carcinomas (82).
In imaging, the tumour shows up in various forms. Intrathoracic lymphadenopathy or a pleural effusion is not very common in this type of cancer. There is a chance to detect tumour calcifications (85).
Histologically the tumour is neuroendocrine in the appearance. Typical signs like trabecular cell growth, palisading structures or rosettes are visible. As the name already indicates, the large cell neuroendocrine carcinoma is characterized by larger tumour cells. The cytoplasm is often mediocre, and the nucleoli are striking.

LCNEC commonly contain large areas of necrosis and often express NCAM, chromogranin or synaptophysin. These neuroendocrine peptides should be used as immunohistochemical markers for diagnosis, and at least one must be positive, in at least 10% of tumour cells (82). In contrast to this, diagnosis of SCLC can be also done without neuroendocrine marker expression, as long as the morphology fits, and other tumours with similar morphology are excluded.

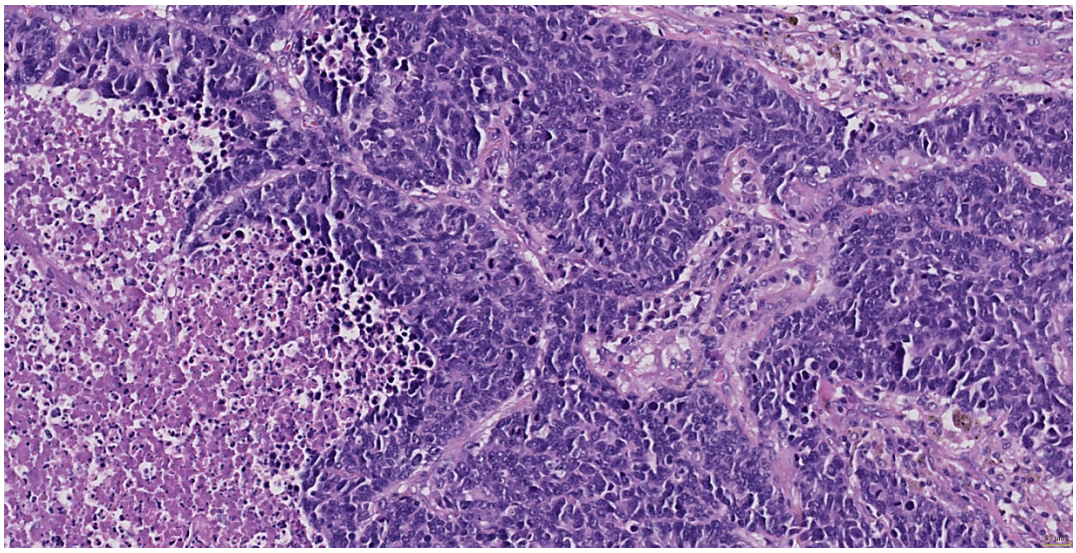

Figure 4: Characteristic growth pattern of large cell neuroendocrine carcinoma.

The prognosis of the LCNEC is poor, but slightly better than the one of the SCLC. The chance of surviving 5 years with an early stage of the disease is around 33% (44) (45).
A recurrence and metastases are more common than in other NSCLC (46). The metastases first spread into the regional lymph nodes, then into the lung itself, followed by haematogenic distribution into brain, liver or bones (40).

1.4.5.3 Carcinoid tumour

Typical and atypical carcinoids also belong to this group of neuroendocrine tumours. With a low mitotic activity (less than 2 mitoses per 2 mm^2) and no necrosis, the typical carcinoid is considered a low-grade malignancy. The atypical carcinoid on the other hand, can have tumour necrosis, has a higher mitotic activity (2-10 mitoses per 2 mm^2), and belongs to the intermediate-grade malignancies (86).

Carcinoid tumours of the lung generally have a very low incidence (1.5/100.000), which is less than 1% of the all lung cancers. The better differentiated typical carcinoids make up nearly 90% of this group (87) (88) (89) (90).
Younger people and especially women have a higher risk of developing this type of tumours (91).

They occur in combination with diffuse idiopathic pulmonary neuroendocrine cell hyperplasia, which is believed to be the preinvasive or precursor lesion of carcinoids. However, they are not associated with tobacco smoking (86).

The carcinoids usually appear near the bronchus, but about 30% of these tumours are in the periphery. In some cases, a neuropeptide production is reported, which can lead to acromegaly or a carcinoid syndrome. Due to the production of adrenocorticotropic hormones a Cushing syndrome can occur (86).

Typical and atypical carcinoids have slightly different prognosis: atypical carcinoid is generally associated with a poorer survival when compared with typical (92) (93). An older age, lymph node metastases and strong cigarette consumption deteriorate the prognosis additionally (93). The 5-year survival rate for typical carcinoids is nearly 90%, while a patient with atypical carcinoid has a chance of 60% to survive the next 5 years. A surgical resection improves the prognosis (94) (95).

1.5 Genetics

Neuroendocrine lung tumours are genetically inconsistent. As mentioned above, the typical and atypical carcinoids (low and intermediate grade tumour) often appear in combination with a multiple endocrine neoplasia syndrome type 1 or an ordinary MEN1 mutation. This aberration is never found in the high-grade malignancies and appears more often in atypical than in typical carcinoids (72) (73).
In the high-grade tumours (SCLC and LCNEC) the mutation rate is very high and the main genetic lesion is the inactivation of the main tumour suppressor genes TP53 and RB (82) (68) (96).
The elimination of TP53 leads to an allelic imbalance (97). The consequence is the loss of the chromosome 3p, 4q, 5q, 13q and 15q (98). Especially chromosome 3p is very important, as it includes many of the human tumour suppressor genes like FHIT, FUS1, VHL or RASSF1 (99) (100) (101). Furthermore, there is a large number of both, epigenomic and genomic lesions in neuroendocrine lung carcinomas, which unfortunately cannot yet be used for therapy (102) (103) (104).

1.6 DLL3, RB, p53

1.6.1 RB/p53 pathway

The RB (Retinoblastoma) gene and the p53 gene are the two main tumour suppressors in the human organism and switched off in many of the most common malignancies, including SCLC and LCNEC (105)(106).

RB gene is located on the 13th chromosome (13q14.1-q14.2) and encodes the RB protein (105). The main function of this tumour suppressor is to stop the cell cycle in the G1 (first gap) phase. It inactivates the transcription factor E2 promoter-binding–protein-dimerization partner (E2F-DP), which is very important for the transition to the S phase. Therefore, the cell cannot reach the S (synthesis) phase and because of that, DNA replication is not possible.
This prevents the human body from excessive cell growth and the development of cancer. Only if the cell is ready and no damages to the DNA are registered, RB is inactivated through phosphorylation by cyclin-dependent kinases (CDK) (107) (108). However, once the gene is permanently inactivated by mutation, tumours occur much more frequently. If this occurs already in childhood, a highly malignant retinoblastoma will develop (109).

The p53 gen, often called “the guardian of the genome”, was found in the 17th chromosome (17p13.1) (110). It has many different functions and is important for the correct course of the cell development. It activates DNA repair if the DNA is damaged, or initiate apoptosis if the damage is irreversible (111) (112). Another function is to stop the cell cycle in the G1 phase (through the cyclin-dependent kinase inhibitor p21 [also called WAF1]), to allow the DNA repair proteins to do their job and start the cell cycle again. If there is no damage registered, p53 remains inactivated by the Mdm2 protein, which reacts with the tumour suppressor (111) (112) (113) (114). However, if p53 is permanently inactivated by mutation, like in an RB mutation, tumours appear much more frequently. There are also syndromes associated with a loss of the RB gene called Li-Fraumeni syndrome or sarcoma, breast, leukaemia and adrenal gland (SBLA) syndrome. (115).
On the other hand, typical carcinoids and tumorlets are not associated with a mutation of p53. However, atypical carcinoids are associated with p53 loss in around 11% (116).

1.6.2 DLL3

Another pathway leading to neuroendocrine lung cancer, beside the already mentioned RB/p53, is the NOTCH-ASCL1-RB-p53. This axis contributes to an alternative secondary pathogenesis in contrast to the RB/p53 pathway, which seems to be responsible for the primary development of the neuroendocrine carcinomas (117). Often found in SCLC, the achaete-scute homolog 1 (ASCL1) leads to increased dissemination and increased growth. Therefore, it interacts with and upregulates the cyclin-dependent kinase 5 (CDK5), which phosphorylates RB and inactivates it (117) (118).

ASCL1 itself on the other hand is suppressed by the neurogenic locus notch homolog (NOTCH) (119). In many different neuroendocrine tumours, a NOTCH mutation is also commonly found (120). Furthermore, NOTCH is inhibited by DLL3 (delta like 3 protein), which belongs to the group of NOTCH-ligands (121). The DLL3 protein physiologically has an important role in the development of the central nervous system, but is also upregulated in neuroendocrine carcinomas, especially in SCLC, which offers hopeful possibilities for future therapies (122) (123).

1.7 Therapy

The different subtypes of lung cancer have made different therapeutic progress in recent years. Treatment of NSCLC depends on the stage of diagnosis (124) (125).
The favored therapy in patients with a very early stage of NSCLC is surgery or stereotactic radiotherapy, while patients with an early to intermediate stage get additional chemotherapy and radiation treatment. Furthermore, patients with an extensive disease stage get either chemotherapy or immunotherapy depending on the genetic mutations (126) (127) (10).
First line treatment of NSCLC without the expression of EGFR, ALK or ROS1 aberrations and an expression of PD-L1 (>50% of the tumor cells), regardless of the histology, is an immunotherapy with the PD-1 antibody Pembrolizumab (128) (10).
NSCLC with a squamous epithelium histology and an expression of PD-L1 of <50% should get a combination therapy of a platinum based chemotherapy, (Pembrolizumab and Paclitaxel) while non-squamous epithelium NSCLC should be treated with a combination of a platinum based chemotherapy (Pembrolizumab and Pemetrexed). (129) (10)
Targeted treatments for non-squamous NSCLC with activating EGFR mutations are tyrosine kinase inhibitors (like Osimertinib or Afatinib). Tumors with ALK-translocation will also receive targeted therapy (for example with Alectinib), while the treatment of a NSCLC with a BRAF-mutation consists of a combination of Dabrafenib and Trametinib. Crizotinib is the drug of choice for the first line treatment of NSCLC with ROS1-translocation. (10) (125)

Despite the success of immunotherapy in NSCLC the treatment of SCLC has not made the same improvements. Local treatment like resection or radiation are only options in very early stages (very limited disease VLD). Patients with Limited disease of SCLC (LD) are treated with a combination of Chemotherapy and Radiation. Nevertheless, about 70% of patients with SCLC have metastases at time of diagnosis (extensive disease ED). Chemotherapy and survival have made very little progress in the last four decades. Treatment in this stage includes a combination-chemotherapy with a Platin (Cisplatin or Carboplatin) and mainly Etoposide. Response rates are high (70%) but disease recurrence is very frequent (130) (131) (132).
Unfortunately, the disease quickly adapts to the drugs and a relapse occurs relatively frequently. If that´s the case, most therapies are limited. Treatment options in the second

line is a re-challenge of the Platinum-combination (for chemotherapy sensitive patients with a relapse later than 6 month) or Topotecan if the relapse occurs earlier. (133) (134) (135). However, the DLL3-inhibitor Rovalpituzumab tesirine provides a new, hopeful therapeutic approach (136).

1.7.1 Rovalpituzumab tesirine

Rovalpituzumab tesirine, antibody-drug conjugate targeting DLL3, has finished open-label phase 1 study. It is composed of a monoclonal IgG1 antibody called SC16 and the SC-DR002, which is a cross-linking agent of the DNA. In addition, to combine SC16 to SC-DR002, a special protease is added. The study, that has first investigated Rovalpituzumab tesirine, included 82 participants. 74 people with SCLC as well as eight patients with LCNEC were treated between 2013 and 2015 at ten US cancer centres. Every participant got at least one dose of the drug. Toxic dose-limiting effects, like a heavy thrombocytopenia and liver disorders appeared in patients who had been given the drug at a dose of 0,8mg/kg (every three weeks) (136). In addition to that, increased lipase and pleural effusion was registered in some cases. However, at a lower dosage of max. 0,4mg/kg (every three weeks) these side effects could be observed much more rarely. At this dosage, an objective therapeutic reaction could be demonstrated in about 18% of the participants. Furthermore, 54% had stable disease. The patients who showed a high DLL3 expression (more than 50% of tumour cells positive) showed a response in 38% of the cases and 88% had a stable disease. In the whole cohort, the average time of response was 5.6 months and the progression free survival 2.8 months, while the high expressed DLL3 cases showed a shorter time of response (4.3 months) but a longer period of progression free survival (4.3 months). Participants with low expression of DLL3 on the other hand showed no response and a progression free survival of only 2.2 months.
The research concluded that the DLL3-inhibitor Rovalpituzumab tesirine has an antitumor activity at a justifiable toxicity. Therefore, another clinical study is recommended (136).

2 Material and Methods

2.1 Patient Cohort

Main study cohort included 53 surgically removed high-grade neuroendocrine lung carcinomas, in the period from 1996 to 2012, with available paraffin blocks, which were provided by the Lung Archive, Institute of Pathology, Medical University of Graz. All available slides were again analysed, in order to validate the diagnosis and choose the most appropriate slide to construct the tissue microarray (TMA). Cores of 1 mm in diameter were made from each tumour (two to seven cores per tumour), including one with normal (non-tumour) lung parenchyma.

Additionally, 46 SCLC tumour samples were chosen as a validation cohort for the small cell lung cancer samples analysed in the study cohort. This group consisted of 12 resection samples and 34 transbronchial biopsies.

The present study was approved by the Ethic Committee of the Medical University of Graz (EK-Nummer 24-135 ex 11/12).

2.2 Immunohistochemistry

4-µm sections were cut out of every TMA, as well as every paraffin block in validation cohort and fixed on positively charged glass slides. Four antibodies were used. As a referral antibody DLL3 ready-to-use assay (clone SP347, Ventana, Roche, Tucson, AZ, USA) (VenA), after 80 min pre-treatment with CC1, and using OptiView detection kit (Ventana) on Benchmark Ultra slide staining instrument (Ventana) was used.

Additionally, three different polyclonal DLL3 antibodies were tested: PA5-26336 (1:150; Thermo Fisher Scientific, Waltham, MA, USA (TherA)); NBP2-24669 (1:150; Novus Biological, Littleton, CA, USA, (NovA)); and ab103102 (1:500; Abcam, Cambridge, MA, USA (AbcA)). DAKO Autostainer (DAKO, Glostrup, Denmark) was used for staining, after 40 min pre-treatment with MW 9.0 at 150 Watt. For detection, EnVision Kit 5007 (DAKO, Glostrup, Denmark), with DAB was used.

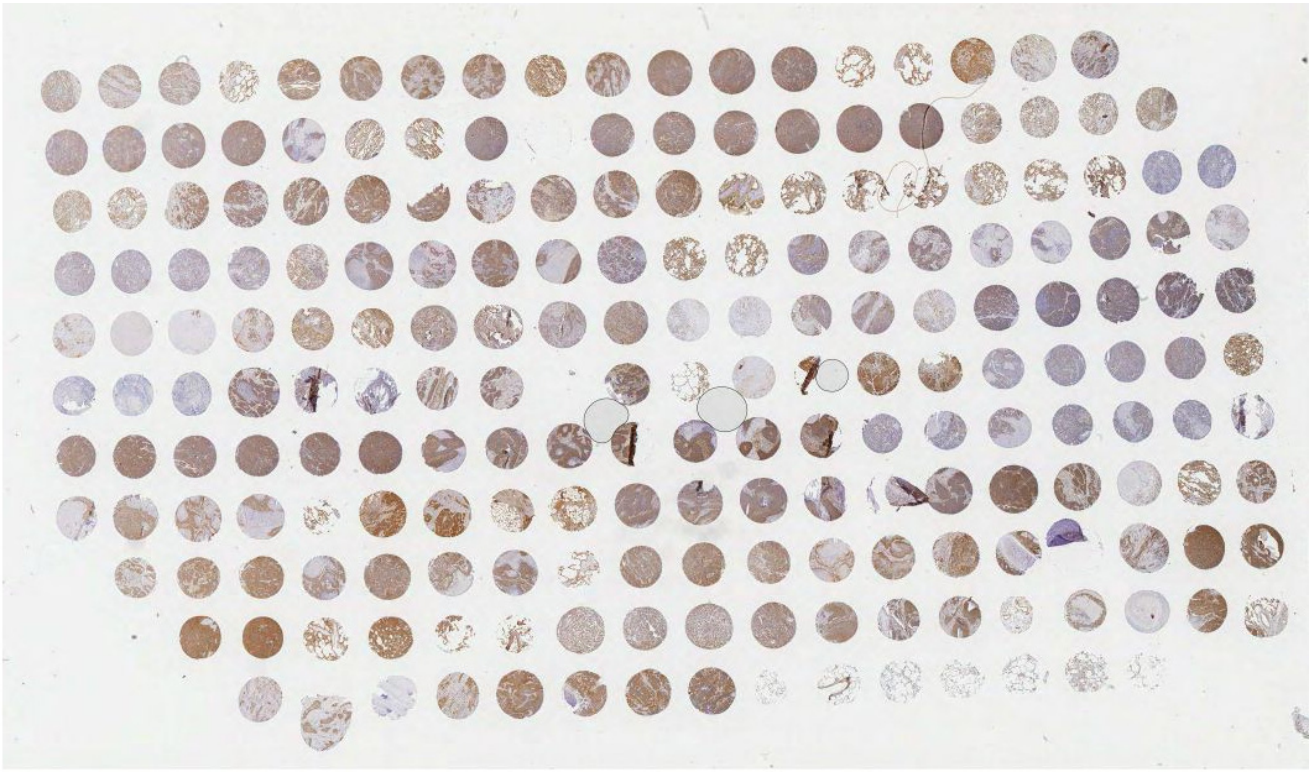

Figure 6: Overview of TMA stained with the AbcA antibody.

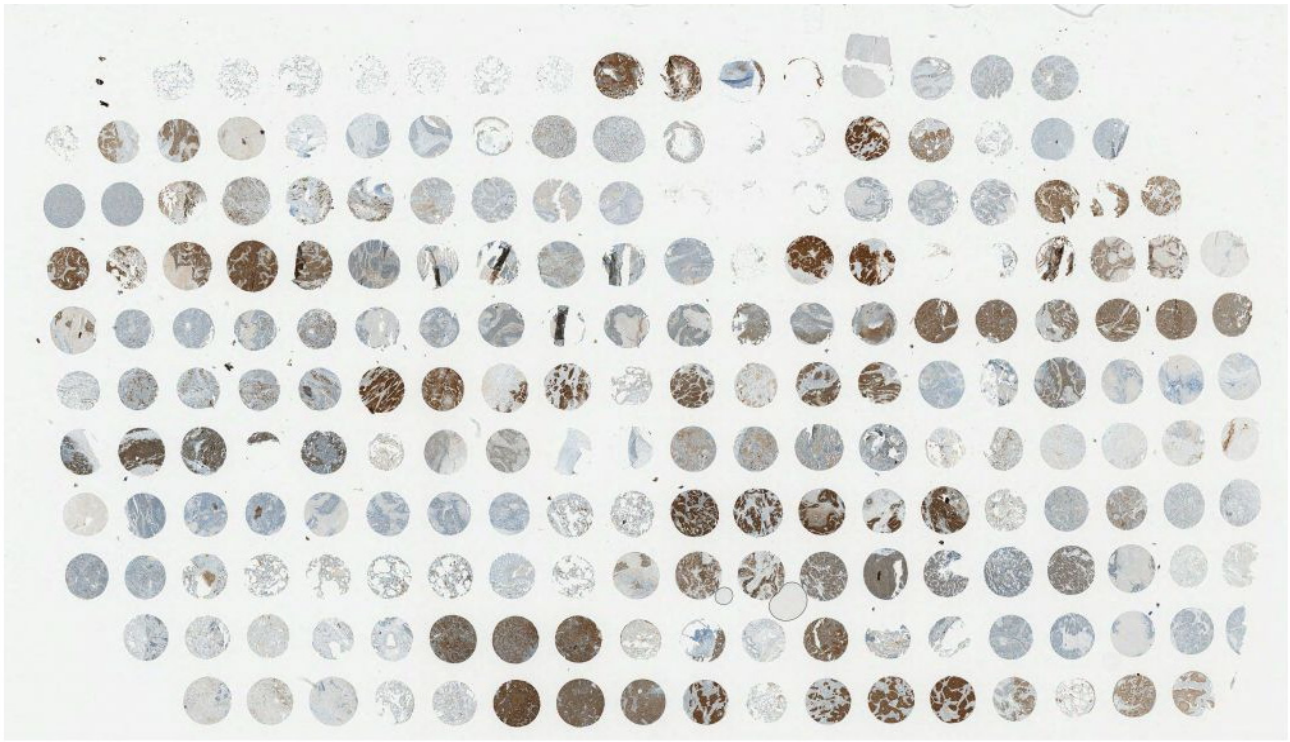

Figure 5: Overview of the TMA stained with the VenA antibody.

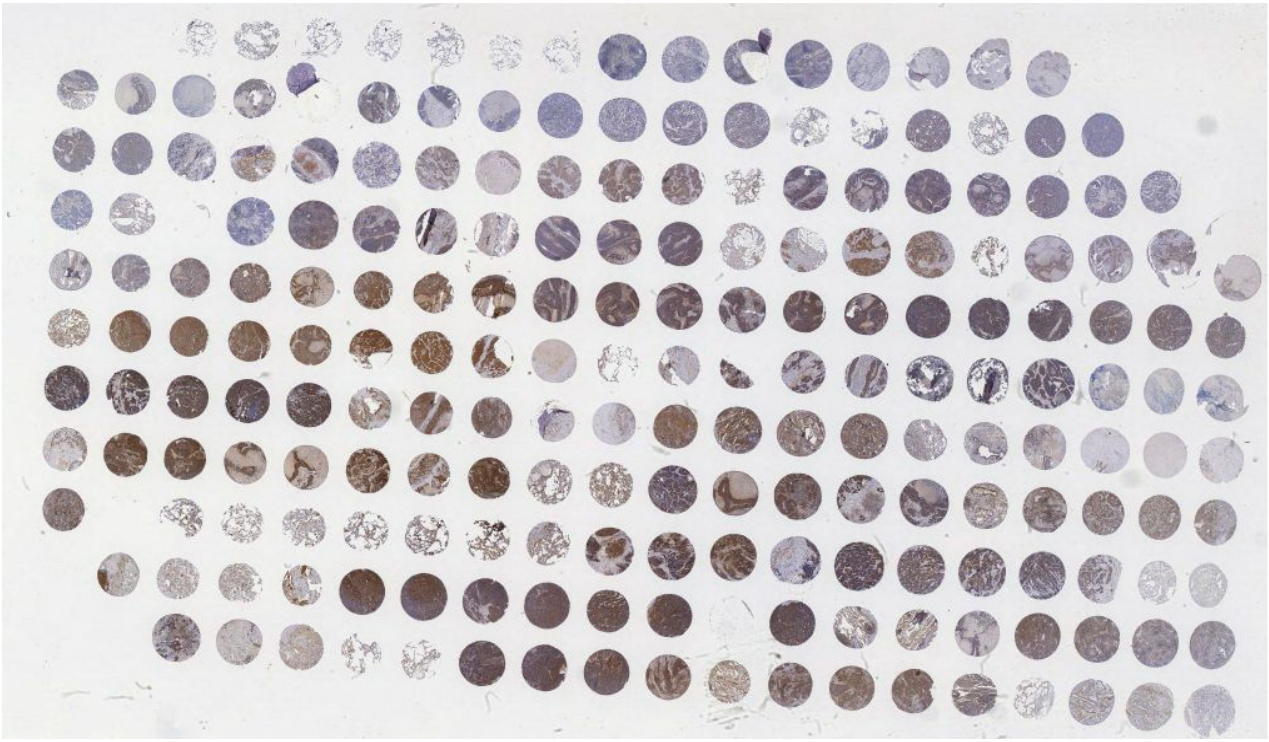

Figure 7: Overview of the TMA stained with the NovA antibody.

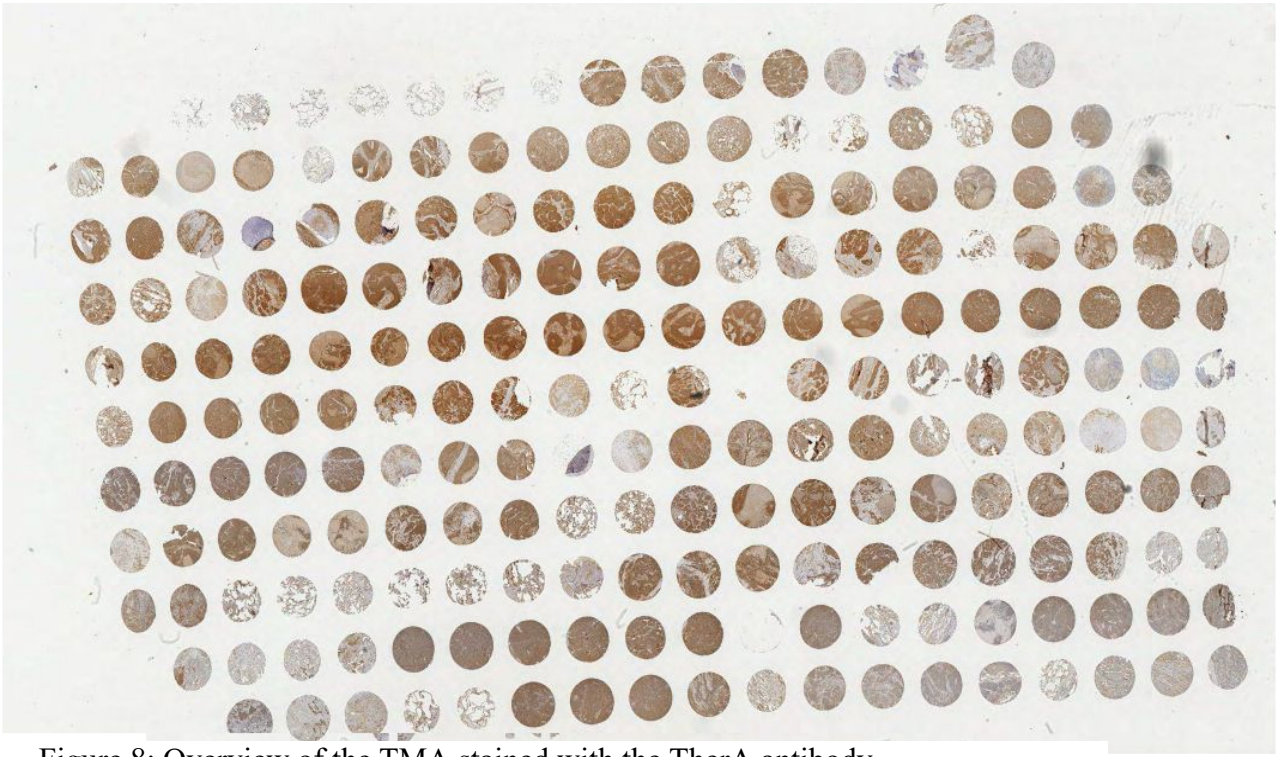

Figure 8: Overview of the TMA stained with the TherA antibody.

DDL3 antibodies were regarded as positive reaction. The intensity of the staining using three-tiered system (3-high, 2-intermediate, 1-low) as well as the percentage of positive tumour cells were assessed under microscope and recorded. Intensity multiplied by percentage of positive tumour cells (H-score) was calculated for each core and expressed as average for each tumour. Disparate and difficult TMA´s were discussed together and an agreement was reached for each case.

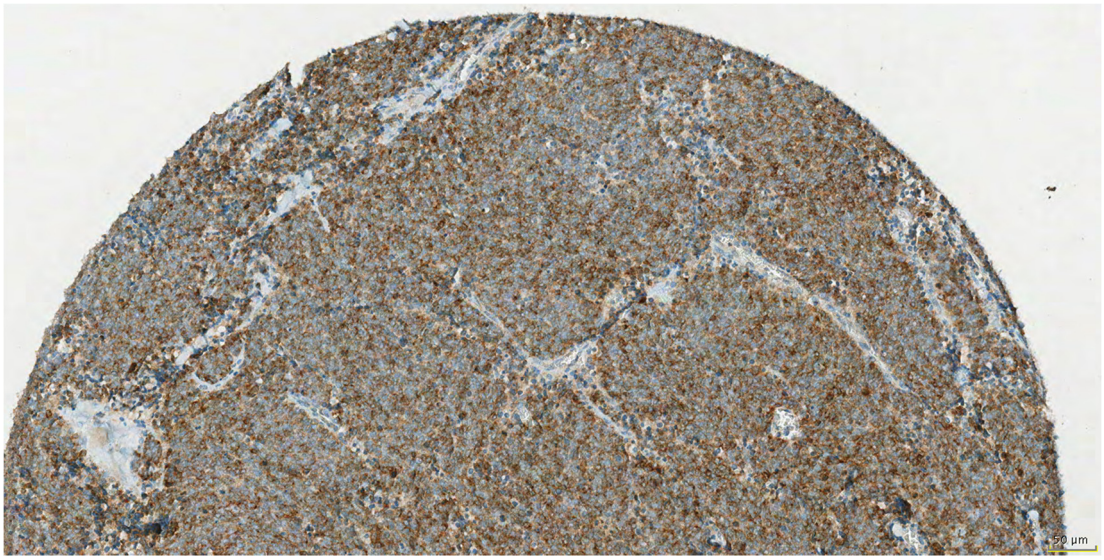

Figure 9: DLL3 staining using reference antibody: positive reaction in SCLC. (Immunohistochemistry, objective x10)

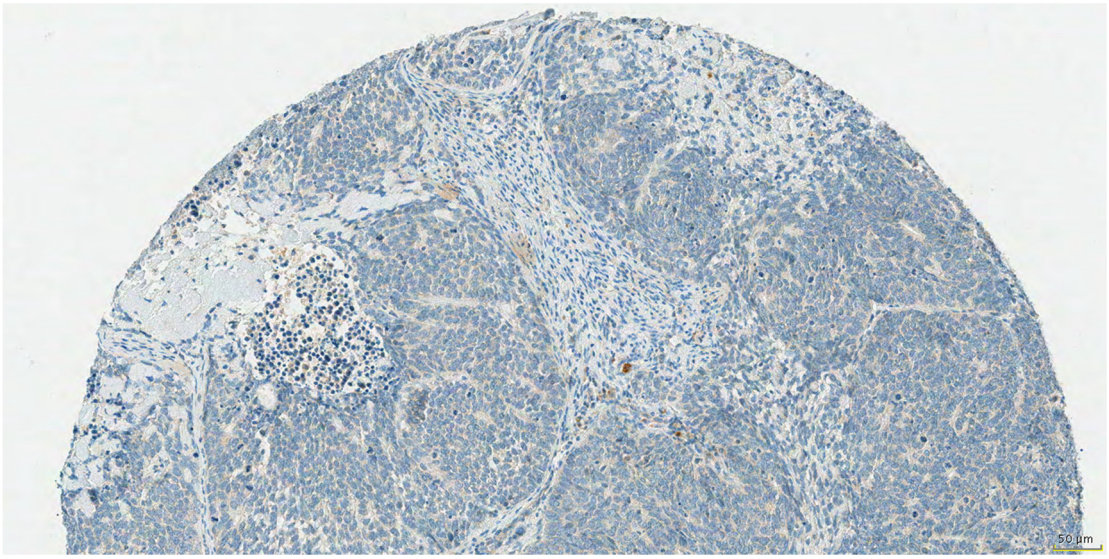

Figure 10: DLL3 staining using reference antibody: negative reaction in SCLC (Immunohistochemistry, objective x10)

2.3 Statistical analysis

Percentages and medians were used to represent the data. Cohen's kappa was used to evaluate agreement between antibodies regarding DLL3 expression. The Spearman correlation coefficient was used to objectify the agreement on H-scores and percentage of DLL3 expressing positive cells. To calculate the outcome the statistical software R 3.5.1 (www.r-project.org) was used.

The significance threshold for the statistical tests was $P < 0.05$.

3 Results

Patient cohort

The main study group included 24 SCLC, and 29 LCNEC. In the SCLC group, there were 20.8% (5/24) women, and 79.2% (19/24) men. In the LCNEC group, the majority were also men, including 62.1% (18/29), with 37.9% (11/29) women. Median age for the SCLC group was 66 years (range 50-86), while the median age in the LCNEC group was also 66 years (range 37-79). Smoking status was known only in 24 patients. 18 of them were smokers, 6 didn´t smoke.

The median survival time in the SCLC group was 19 months (range 5 days to 16 years). LCNEC group had longer median survival time (83 months, range 12 days to 25 years). Validation cohort consisted only of SCLC. Majority were males, 80.4% (37/46), with a median age for the whole cohort of 65 years (range 45-78). Follow-up for the validation cohort was not recorded, since this group was used as a validation cohort for staining with different DLL3 antibodies.

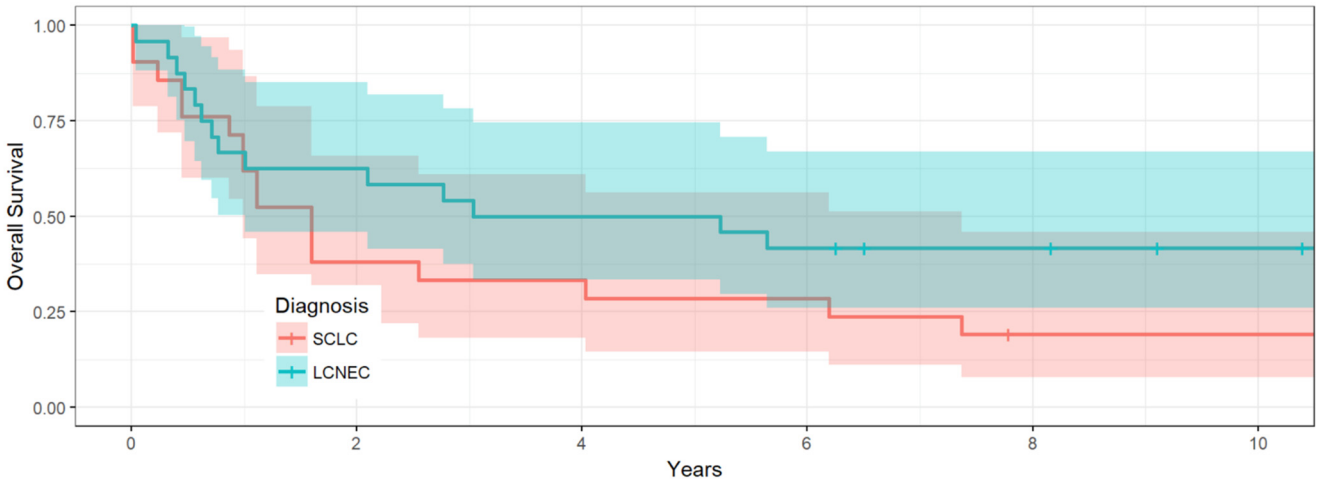

Figure 11: Survival analysis of the main study cohort demonstrated poor survival for patients with both, LCNEC and SCLC.

Comparison of a DLL3 expression of 4 different antibodies in TMA

Both in LCNEC as well as SCLC, a positive expression of DLL3 was present.

In the study cohort, the AbcA and the TherA showed a higher percentage of DLL3 expressing tumour cells, as well as a higher H-score, in comparison to the other two

antibodies, NovA and VenA. In an additional analysis of each subgroup (SCLC and LCNEC) the same result was shown.

Furthermore, comparing each antibody separately, major differences were present in terms of tumour cells positivity.

In SCLC cells the reference antibody VenA showed a staining of more than 75% of the tumour cells in 5/24 (20.8%) of the cases. For ≥50% 11/24 (45.8%) cases showed an expression and at a cut-off value over 25% 14/24 (58.3%) of the samples were positive.

In the other three antibodies, NovA, TherA and AbcA the distribution results for DLL3 expressing lung cancer cells were, ≥75%: 5/24 (20.8%), 7/24 (29.2%), 5/24 (20.8%), ≥50%: 12/24 (50%), 18/24 (75%), 14/24 (58.3%), and ≥25%: 20/24 (83.3%), 22/24 (91,7%), 22/24 (91.7%), respectively.

In LCNEC, tumour cells with a cut-off value of over 25% for DLL3 expressing positive tumour cells differences were also present. The reference antibody VenA showed a positive expression in 14/27 (51.9%) of the cases, while NovA, TherA and AbcA were positive in 15/27 (55.6%), 25/27 (92.6%), 24/28 (85.7%) of the samples.

Comparing the reference antibody with the other three antibodies, the overall consent in percentage of positive lung cancer cells ranged from a minimum of 45.8% to a maximum of 83.3%. With a higher cut-off value for positivity (≥25, ≥50, ≥75), the positive agreement rate increased significantly, while the negative agreement rate declined with the rise in cut-off value and was high at the cut-off point of 25%.

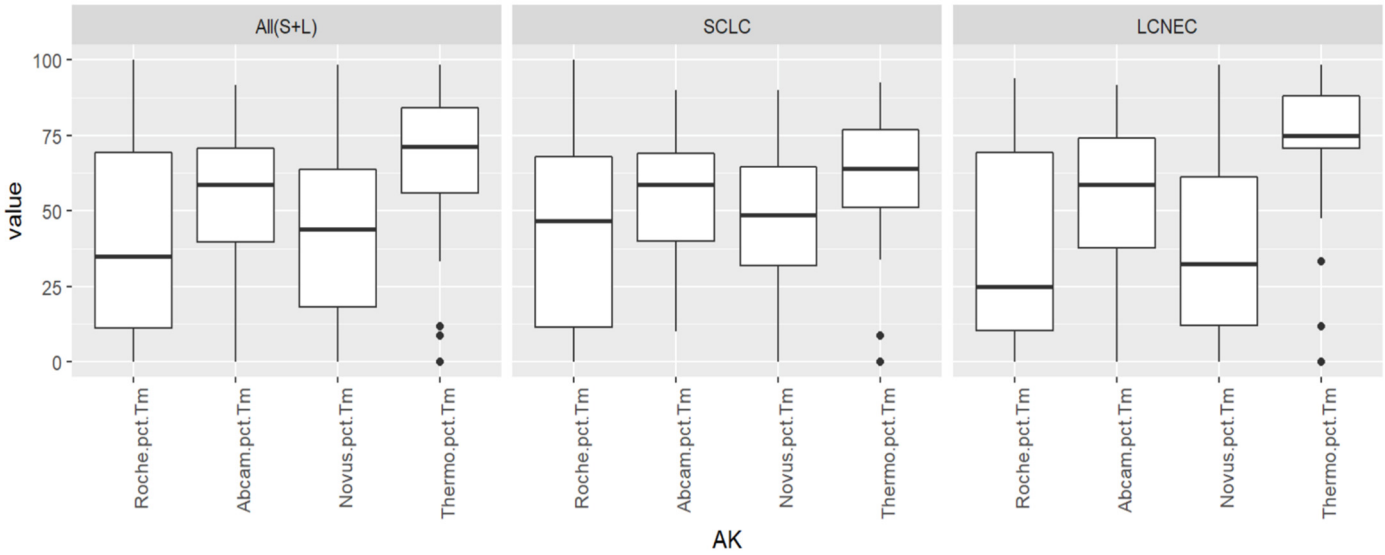

Figure 12: Comparison of the percentage of DLL3 positive tumour cells (pct.Tm) obtained with 4 different antibodies. Results are presented for the whole study cohort, as well as separated for SCLC and LCNEC. None of the antibodies used, showed positive correlation with other tested antibodies.

Comparison of a DLL3 expression of 4 different antibodies in validation cohort

TherA and NovA antibodies showed a lower percentage of DLL3 expressing tumour cells as well as a lower H-score, in comparison to the other two antibodies, VenA and AbcA. At a cut-off of more than 75% of the tumour cells being positive TherA and NovA showed an expression in 20/46 (43.5%) and 10/46 (21.7%) of the cases. For ≥50%, it was 5/46 (10.9%) and 27/46 (58.7) of the cases. At the cut-off values of over 25% the expression was 21/46 (45.7%) and 15/46 (32.6%), respectively. The two better performing antibodies, VenA and AbcA, showed a positive reaction in 15/41 (36.6%) and 13/46 (28.3%) of the cases at a value of ≥75%. For ≥50%, VenA and AbcA showed 32/41(78%) and 32/46 (69.6%), whereas the expressions for ≥25% were 34/41 (82.9%) and 42/46 (91.3%) of the cases, respectively.

In conclusion, the validation cohort showed similar outcome as the main study cohort. In comparison of the three other antibodies to the VenA, which was used in the clinical study, they showed poor results in terms of positive and negative agreement, as well as concerning Kappa values and overall agreement.

In case by case analysis, TherA showed statistically significant difference in 20/46 samples and NovA in 17/46 cases, even being positive in cases, where the reference antibody demonstrated negativity. AbcA differed in 8 out of 46 cases as well: the reference antibody VenA was negative in four positive cases and only slightly positive in another negative case.

Correlation of RB1 and TP53 expression with DLL3 expression in SCLC and LCNEC

The relationship between the RB1/TP53 loss and a DLL3 expression was also investigated in the main study cohort. Only reference antibody (VenA) was used in this analysis.

There was no statistically significant correlation between the expression of DLL3 (VenA) and the loss of RB1 and TP53 in the large cell neuroendocrine carcinoma.

Similar results have been found in the SCLC group for TP53, but there was a statistical significance of RB1 loss in small cell lung cancer and DLL3 expression.

SCLC showed an expression of TP53 in 15/21 (71.4%) cases and an expression of RB1 in 9/24 (37.5%) cases.

LCNEC, on the other hand, expressed TP53 in 15/28 (53.6%) cases and RB1 in 16/27 (59.3%) times.

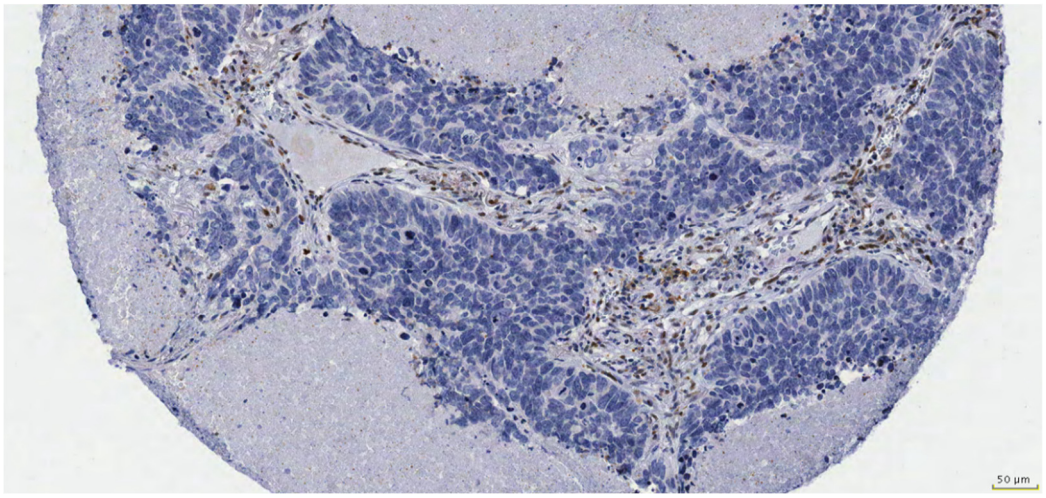

Figure 13: Presentation of the expression of the antibody against RB1: Loss of RB1 in SCLC (Immunohistochemistry, objective x10)

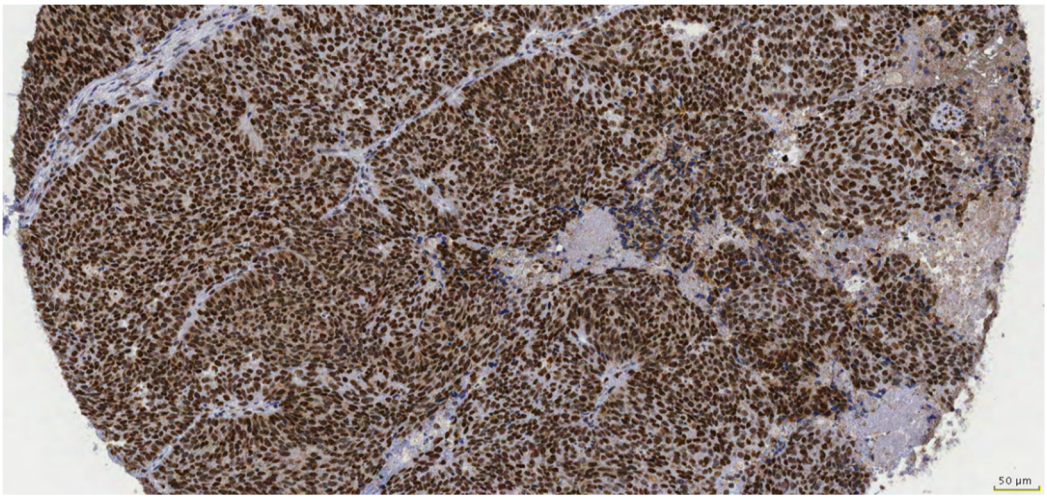

Figure 14: Presentation of the expression of the antibody against RB1: Positive nuclear reaction in SCLC (Immunohistochemistry, objective x10)

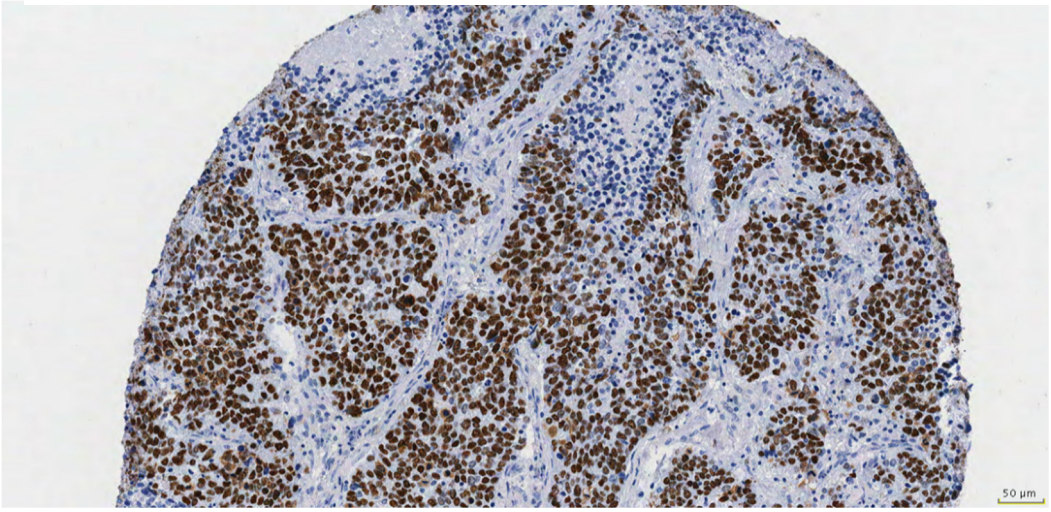

Figure 15: Presentation of the immunohistochemical staining with the antibody against TP53: nuclear positive reaction in SCLC (Immunohistochemistry, objective x10)

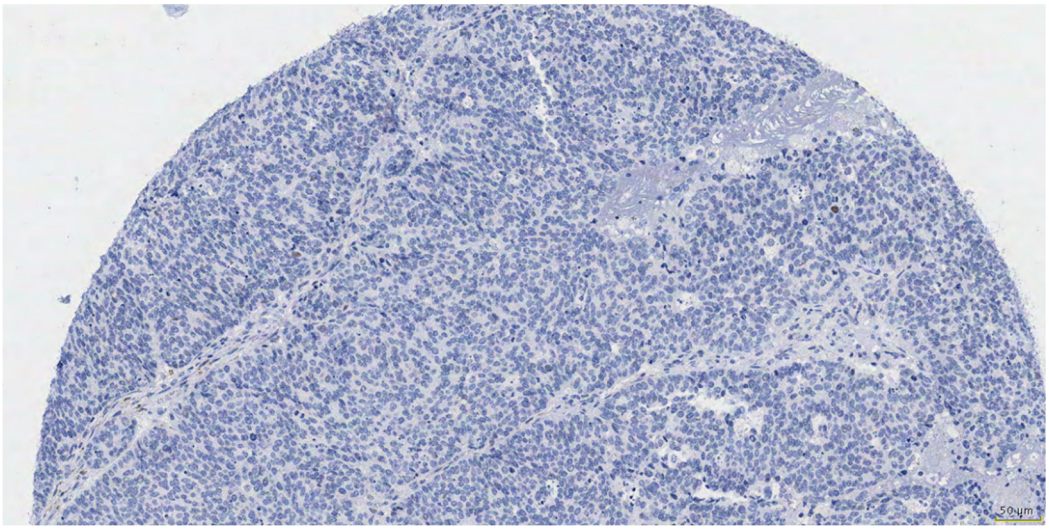

Figure 16: Presentation of the immunohistochemical staining with the antibody against TP53: complete negative reaction in SCLC (Immunohistochemistry, objective x10)

Beyond that, ranging from -23.2 up to 33.9, Kappa values were extremely deficient and neither the percentage of DLL3 expressing tumour cells, nor the H-score of all tested antibodies showed correlation with the overall survival. Furthermore, the expression of RB1 and TP53 showed no correlation with the survival.

These results apply to both SCLC and LCNEC, separately, as well as to the entire TMA study cohort.

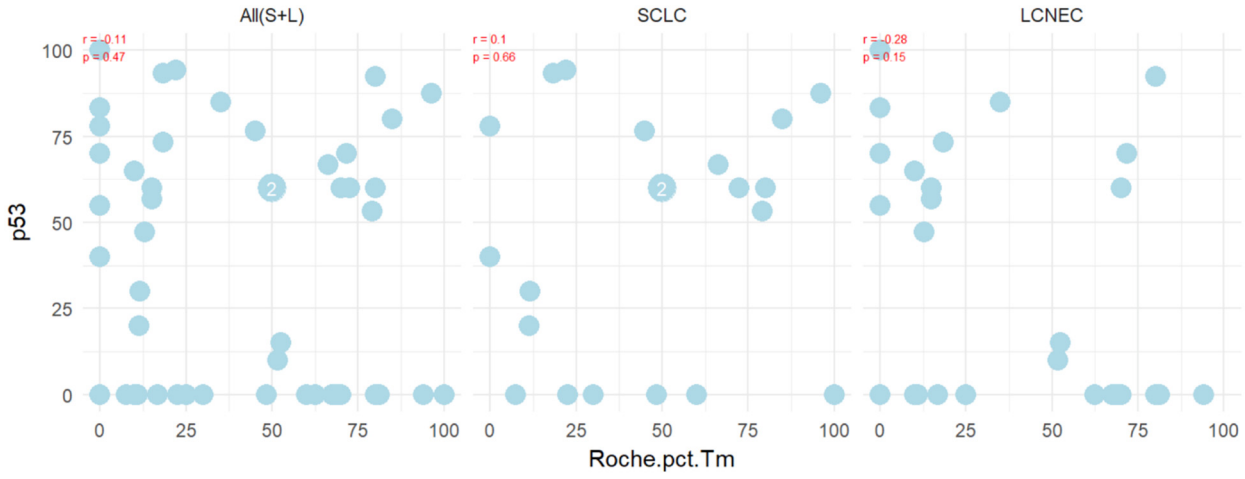

Figure 17: Correlation of DLL3 positive tumour cells (Roche, pct.Tm) with the expression of p53 using the reference antibody did not reveal any statistically significant relation.

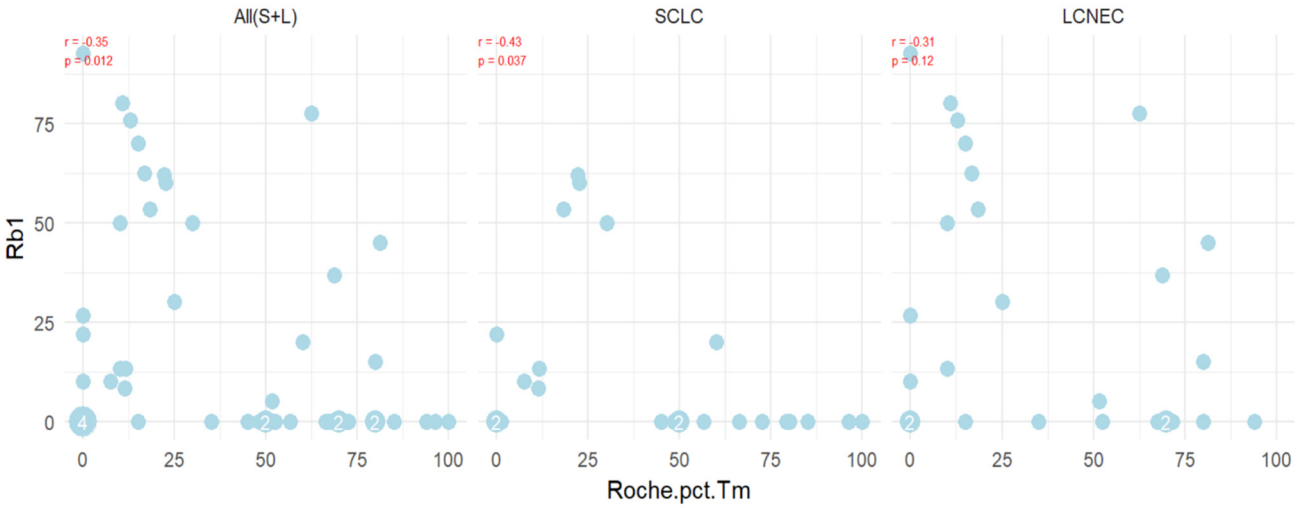

Figure 18: Analysis of the correlation of DLL3 positive tumour cells (Roche, pct.Tm) with RB1 loss using the reference antibody demonstrated statistically significant results in SCLC, but not in the LCNEC nor when the group was analysed as a whole.

4 Discussion

Lung cancer is still one of the most prevalent forms of cancer in the modern society and remains at the top of the cancer statistics in men (137). In women, mostly due to the different amount of tobacco smoked, lung cancer is at the second place, right after breast cancer (2).

The small cell lung carcinoma makes up about 13% of the lung cancers recorded every year. It´s almost exclusively associated with heavy tobacco smoking (64).

Although in the last decade huge improvement in therapeutic options for adenocarcinoma, as well as for squamous cell carcinoma is obvious, no significant change in the therapy of small cell lung cancer has occurred so far. Currently, a phase 1 study of an antibody-drug conjugate which targets DLL3 is published, with promising results. This first-in-class, first in human study of a DLL3-targeted antibody-drug conjugate can lay the foundation for the future therapy of people suffering from a small cell lung carcinoma (136). This would mean a major evolution in the therapy of these patients, who commonly have an advanced disease stage with poor prognosis.

Due to these results and knowing that DLL3 expression on tumour cells is prerequisite for this therapy, we have decided to test the expression of four different DLL3 antibodies, in consecutive slides of the same tumour samples. The idea was to analyse the positivity rate with all antibodies, and to see how much they would differ, keeping in mind that not all pathological laboratories are equipped with staining machines adequate for the antibody used in clinical trial. Based on the previous experiences, the last one being PD-L1 testing, there will be many in-house, or laboratory developed tests. In validation phase of these, it is crucial to see how they correlate to the reference antibody.

Our results clearly demonstrated, that none of the 4 used antibodies is interchangeable. Because of that the results of staining differ significantly. As the VenA antibody is the antibody used in clinical study, we have decided to use it as a reference antibody. At all different cut-off values that were analysed in our study (≥75%, or ≥50%, or ≥25% of positive tumour cells in the sample, with respect to VenA), there was not even one without difference in DLL3 expression between all four antibodies applied.

These disconcordant results were present in both tumour groups, large cell neuroendocrine carcinoma as well as the small cell lung carcinoma group.
We have also investigated the DLL3 expression in LCNEC. If we look at the results obtained with VenA antibody it reveals that roughly 50% of these carcinomas were positive. That implies that LCNEC patients, which express DLL3, could probably benefit from a DLL3-based antibody therapy.

Recently, new LCNEC therapies have been suggested, depending on mutational analysis. In case of a RB1 loss, chemotherapy similar to the one for the SCLC treatment is recommended. On the other hand, patients with a loss of PTEN or a mutation of Pl3KCA would benefit more from a cisplatin based therapy (138) (139). Furthermore, we have shown that in large cell neuroendocrine carcinoma there is no correlation between the DLL3 expression and a loss of RB1. In other words, DLL3-targeted antibody-drug conjugate is another potential therapeutic option, regardless of RB1 status, and RB1 role. In the group of SCLCs, we have found positive statistical correlation of DLL3 expression and a loss of RB. However, it is not sure if this is a real correlation or just a reflection of the fact that most small cell lung carcinoma have a loss of RB1. Like in other studies previously demonstrated, we were not able to find correlation between RB1, TP53, DLL3 and prognosis.
Our study has some obvious limitations: The study cohort is small, immunohistochemical analysis was performed on archive samples and there was no positive and negative control to be included in analysis.
However, by using real-life samples we have demonstrated feasibility of DLL3 expression analysis, and additionally, a big difference in expression between the analysed antibodies, suggesting that for in-house methods for DLL3 testing some other antibodies should be used.

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