Pulmonary Carcinoids

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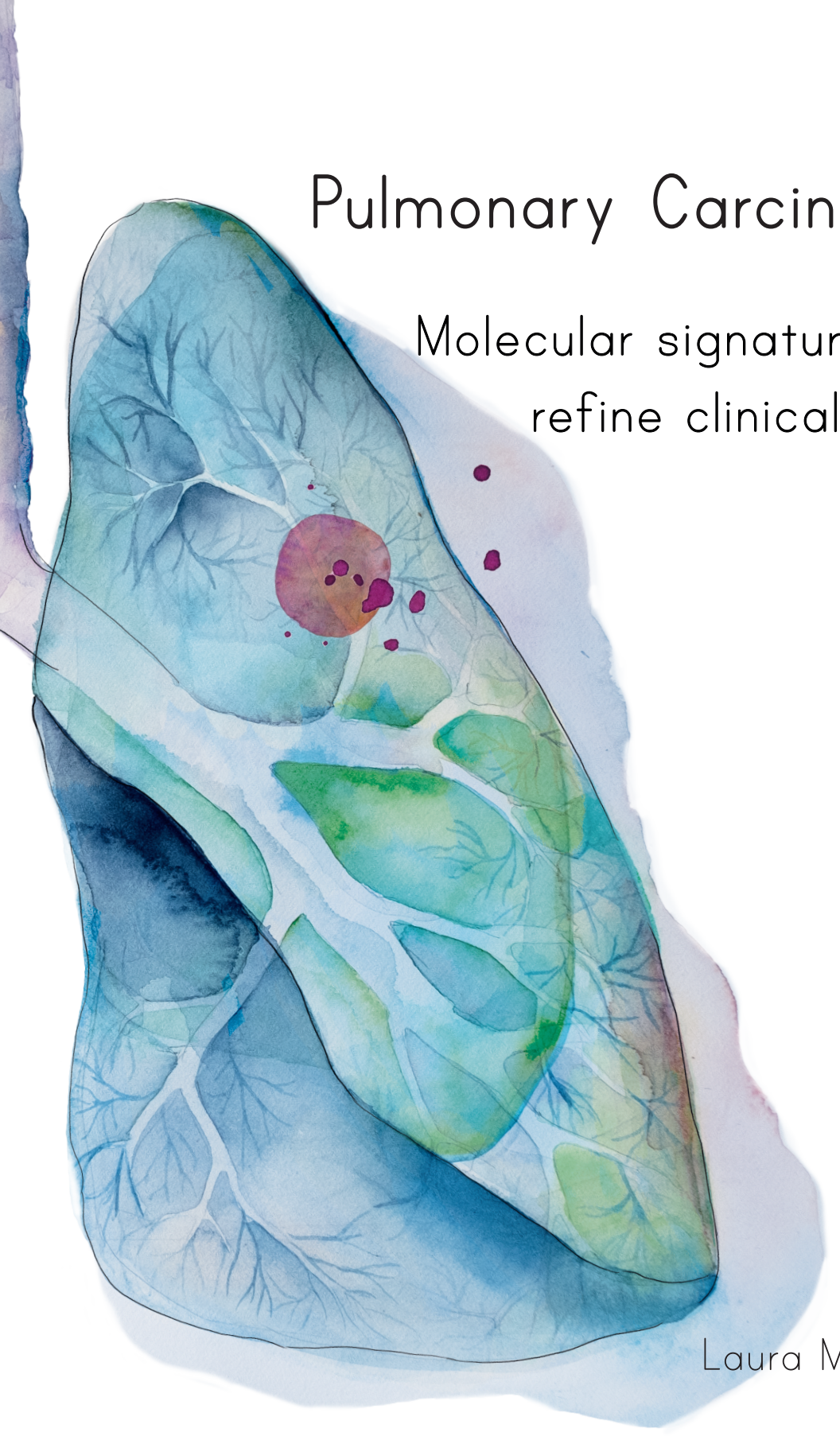
Molecular signatures to refine clinical care



Laura Moonen

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Molecular signatures to refine clinical care

Pulmonary Carcinoids: *Molecular signatures to refine clinical care*
Laura Moonen

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Pulmonary Carcinoids

Molecular signatures to refine clinical care

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Laura Moonen

Promotoren
Prof. dr. E-J.M. Speel
Prof. dr. A-M.C. Dingemans

Co-promotor
Dr. J.L. Derks

Beoordelingscommissie
Prof. dr. A. zur Hausen (voorzitter)
Prof. dr. R. García-Carbonero (Hospital Universitario Doce de Octubre, Madrid, Spain)
Dr. L.E.L. Hendriks
Prof. dr. L.J. Hofland (Erasmus MC, Rotterdam)
Prof. dr. S.W.M. Olde Damink

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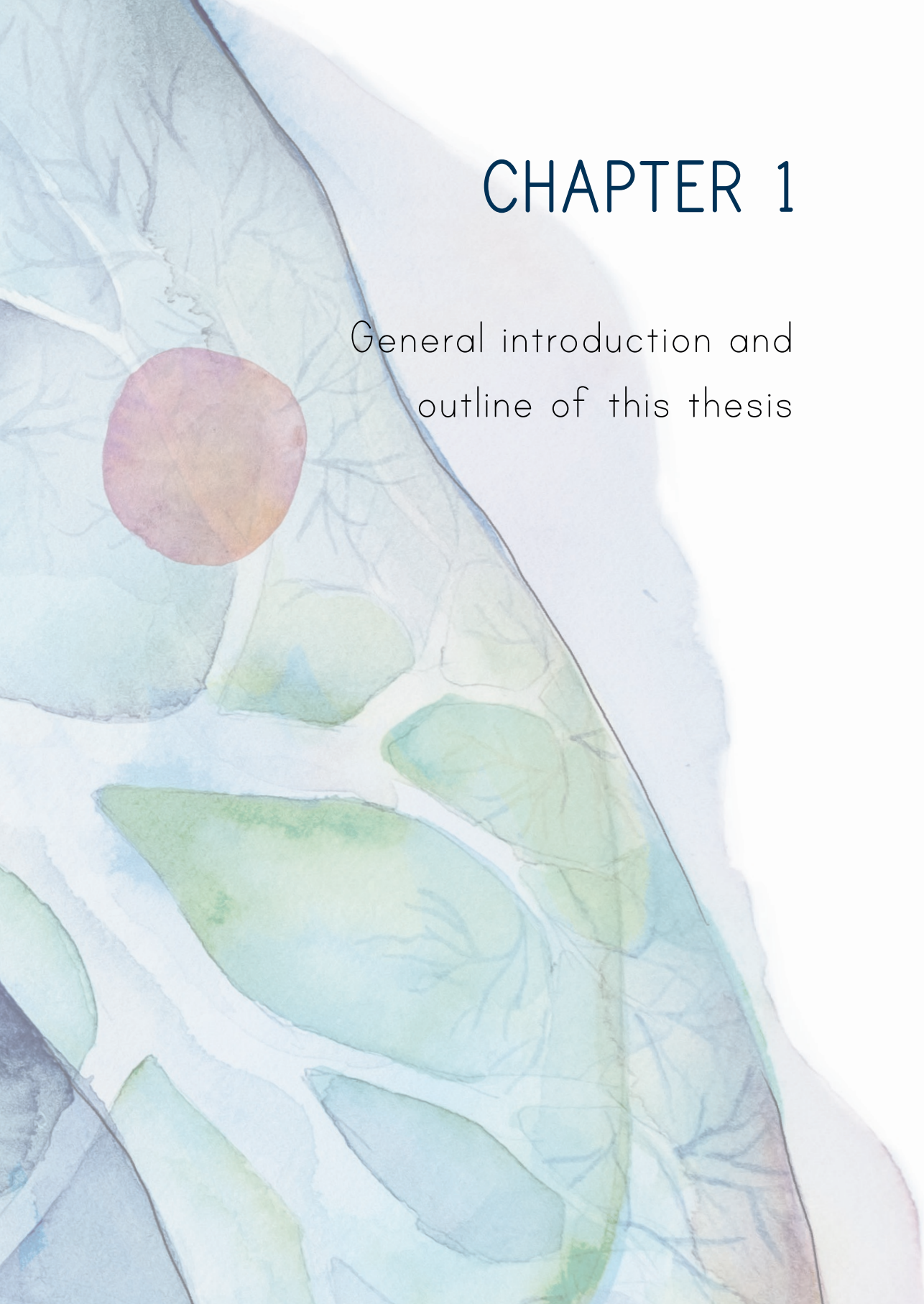

CHAPTER 1

General introduction and outline of this thesis

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Neuroendocrine neoplasms (NENs) represent a group of rare heterogeneous tumors which are characterized by neuroendocrine morphology and differentiation.[1] NENs may develop in various organ systems including intestine, pancreas, prostate, and lung. Pulmonary NENs represent a subgroup of lung cancer, accounting for approximately 15-20%, likely arising from neuroendocrine cells of the bronchopulmonary epithelium.[2] According to the latest World Health Organization (WHO) classification, pulmonary NENs can be classified as neuroendocrine tumors (NETs), comprising low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC), and high-grade neuroendocrine carcinomas (NECs), comprising large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC).[2] 95% of pulmonary NENs are high-grade poorly differentiated NECs, including SCLC (79%) and LCNEC (16%), with carcinoids accounting for only a small proportion (2-5% TC and 0.2-0.5% AC). While the incidence of TC, AC, and LCNEC is increasing among lung cancers, the incidence of SCLC has declined over time.[3-5]

1. Pathological diagnosis of pulmonary carcinoids

The carcinoid tumor was first described as bronchial adenoma by Muller in 1882 and later named Karzinoid by Oberndorfer in 1907.[6] The differentiation between TC and AC was first described in 1944 and Arrigoni *et al*. reported the first histological differentiation criteria for these entities.[7,8] Increased mitotic activity, pleomorphism, prominent nucleoli, hyperchromatism, disorganized architecture, increased cellularity, areas of necrosis were signs of increased potential to metastasize. Travis and colleagues updated these criteria and defined strict WHO criteria in 1999.[9,10] These diagnostic criteria have remained mostly unchanged until present day.

The histopathological diagnosis of pulmonary carcinoids relies on morphological characteristics (e.g. granular ''salt and pepper'' chromatin, lack of prominent nucleoli, organoid nests, trabeculae, rosettes) and the demonstration of the neuroendocrine nature of the tumor through the detection of general neuroendocrine marker immunoexpression (e.g., chromogranin A, synaptophysin and CD56/NCAM).[2] In addition, thyroid transcription factor 1 (TTF1) may be used to prove the lung origin of metastatic tumors in well-differentiated tumors. TCs and ACs are distinguished based on the mitotic index and the presence or absence of necrosis (Table 1.1).[2] TCs have a mitotic index of $<2/2mm^2$, AC of $2\text{-}10/2mm^2$, whereas LCNEC and SCLC have a mitotic index of

>10/2mm^2. In general, necrosis is absent in TC, may be focally present in AC, and is abundant in LCNEC and SCLC. Although most carcinoids have a well differentiated morphology (structured architecture and uniform and round or spindle shaped nuclei) and LCNEC a poorly differentiated morphology (less structured, heterogeneous nuclei), differentiation based on morphology is not included as a criteria in the WHO 2021 classification.[2]

Table 1.1 WHO 2021 diagnostic criteria of pulmonary neuroendocrine tumors.[2]

	TC	AC	LCNEC	SCLC
Mitosis per 2mm^2	<2	2-10	>10 (median: 70)	> 10 (median: 80)
Necrosis	No	Focal, if any	Yes	Yes
NE morphology	Yes	Yes	Yes	Yes
Ki-67 PI	Up to 5%	Up to 30%	30-100%	30-100%
TTF1 expression	Mostly positive in peripheral tumours, mostly negative in central tumors	Mostly positive in peripheral tumours, mostly negative in central tumors	Positive (70%)	Positive (85%)

Abbreviations: TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; NE, neuroendocrine; Ki-67 PI, Ki-67 proliferation index.

Another marker that might support pulmonary NEN classification is the Ki-67 proliferation index (PI). While several studies have reported a prognostic relevance of Ki-67 PI for pulmonary NENs[11-14], other revealed limited prognostic value of Ki-67 in addition to the WHO classification.[15,16] Due to the existence of overlap among Ki-67 cut-off values separating TC from AC, the Ki-67 PI is still not included in the WHO 2021 as a diagnostic criterion for pulmonary carcinoids.[2] Nevertheless, Ki-67 may be useful in pulmonary NENs to distinguish pulmonary carcinoids from LCNEC and SCLC, especially in small biopsy samples.

2. Clinical characteristics

Pulmonary carcinoid disease occurs more often in women than in men.[17] The average age at presentation is around 45 years for TC and 55 years for AC, but carcinoids can also occur at younger age. About eight percent develop in the second decade of life, making carcinoids the most common primary pulmonary tumor of childhood.[18] TC are not correlated to tobacco smoking, whereas AC occurs more frequent in smokers. Pulmonary carcinoids may be located central or peripheral of which TC are more often centrally and AC peripherally located.[2,19-21] Patients with central carcinoids (segmental and larger airways) may present symptoms related to bronchial obstruction such as cough, wheezing, haemoptysis, and pneumonia. Peripheral carcinoids are in general asymptomatic and usually detected as an incidental finding on chest imaging.[2]

Bronchoscopy, transthoracic biopsy, or endobronchial endoscopic ultrasonography (EBUS) are used in the diagnostic work-up. In addition, both fluorine 18-fluorodeoxyglucose (FDG) evaluated by positron emission tomography (PET) scan and 68Ga-DOTATOC PET (targeting the somatostatin 2 receptor) are used to clinically stage carcinoids, of which the 68Ga-DOTATOC PET reaches highest sensitivity (90% versus 71%).[22] In TCs, FDG PET may show increased glucose uptake but lower as compared to AC and other solid lung cancers.[23]

3. Treatment

Surgical resection remains the treatment of choice for loco-regional pulmonary carcinoids, independent of histopathological classification. The surgical approach depends on tumor size, location, and preoperative biopsy specimen assessment.[2] Following European Society for Medical Oncology (ESMO) guidelines, preferred treatment for pulmonary carcinoids is anatomic resection (e.g., segmentectomy, lobectomy, bilobectomy, pneumonectomy) with lymph node resection (with a minimum of six nodal stations: three hilar and three mediastinal, including subcarinal station, as recommend by the European Society of Thoracic Surgery for non-small cell lung cancer).[2] Several studies have reported the noninferiority of parenchyma-sparing resections (e.g., wedge resection and segmentectomy) compared with the traditionally advised lobectomy for TC.[24-28] Based on these studies, the European Neuroendocrine Tumor Society (ENETS) guidelines propose to consider segmental resection (rather than wedge resection) in patients with limited pulmonary function.[3] In addition, ENETS advises to perform parenchyma-sparing surgery for patients with central airway tumors as these are predominantly TC and thus characterized by a lower relapse risk.[3] Nevertheless, the National Comprehensive Cancer Network (NCCN) guidelines still recommend lobectomy or another anatomical resection for TC.[29]

While curative treatment by means of surgical resection is possible for most pulmonary carcinoids, distant disease relapse may occur up to 15 years after primary resection.[3] Recent studies observed distant relapses in 1-6% of patients with TC and 14-29% of the patients with AC after surgical resection of the primary tumor.[30-35] As a result, the ENETS advises up to 15 years of follow-up after primary tumor resection.[3] The Northern American Neuroendocrine Tumor Society (NANETS) advises long-term follow-up for all AC and only for TC with lymph nodal involvement (N1-N3) or for tumors larger than 3 cm, tumors with close tumor resection margins or tumor multifocality.[36] ESMO advises that follow-up should be life-long, and the frequency is dependent on morphological grading and ''clinically slowly vs. rapidly progressing carcinoids''.[17] A TC, pT1-2 with a pN0

status, is regarded as a tumor with low relapse risk and requires less clinical and radiological follow-up as compared to TC with a pT3-4 and pN1-2 status or AC with a pN1-2. Thus, guidelines diverge concerning follow-up recommendations, and follow-up frequency increases based on pathological staging.

Routine adjuvant therapy is not recommended in pulmonary carcinoids. In case of advanced pulmonary carcinoids, possible systemic anti-tumor therapies are somatostatin analogues (Octreotide and Lanreotide), chemotherapy, everolimus, peptide receptor radionuclide therapy (PRRT) and interferon-alfa. However, only everolimus is approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of pulmonary carcinoids.[17]

4. Molecular background

Genomic studies have shown that pulmonary carcinoids generally contain a low mutational burden and only few recurrent mutated genes.[37-41] Overall, the most frequently identified aberration is a mutation in menin 1 (*MEN1*), which is also associated with poor prognosis.[37,39,42] Other frequently mutated genes (*ARID1A*, *EIF1AX*, members of the SWI/SNF complex) are implicated in the chromatin remodelling pathway, a process that controls gene expression. Mutations in oncogenic driver genes such as *TP53* and *RB1* are rare in pulmonary carcinoids.[37,39,43]

Orthopedia Homeobox (*OTP*) has been suggested as a prognostic molecular marker for pulmonary carcinoids, alone or in combination with *CD44*, to distinguish good from poor prognostic patients.[44,45] The prognostic value of *OTP* has since been evaluated in larger series, confirming that loss of expression is associated with poor prognosis.[11,46-50] Nevertheless, since these cohorts were retrospective single/multi-institutional, they represent a selection of patients that often contained limited or incomplete follow-up. Thus, validation of *OTP* and *CD44* in future studies remains relevant. In addition, the molecular characteristics of pulmonary carcinoids should be investigated in more detail in multi-omics studies to unravel the regulatory mechanisms underlying carcinogenesis.[51]

5. Aims and outline of this thesis

In this thesis, 1) the reliability of the histopathological diagnosis and prognosis of patients suffering from pulmonary carcinoid is examined using a unique population-based cohort, 2) the molecular mechanisms underlying carcinoid tumorigenesis are

investigated, 3) molecular biomarkers to improve the prognostication of pulmonary carcinoid patients are identified and verified, and 4) the first patient-derived tumor organoids for pulmonary neuroendocrine neoplasms to uncover therapeutic vulnerabilities are reported.

In **chapter 2** of this thesis, the relation between disease relapse and extent of lymph node sampling in pulmonary carcinoids patients was examined in a population-based cohort (2003-2012). **In chapter 3-4**, we determined, within this population-based cohort, whether final carcinoid classification on a resection specimen can be predicted by a preoperative biopsy. **Chapter 5** provides a literature review addressing the current clinical value of OTP and the possible molecular mechanisms regulating OTP expression and function in pulmonary carcinoids. In **chapter 6** we validated a molecular marker panel by using a matched patient cohort (2:1) for non-relapse *versus* relapse to improve the prognostication of pulmonary carcinoid patients. In **chapter 7** we explore multi-omics data, to unravel the mechanism underlying differential *OTP* expression. **Chapter 8** describes the development and verification of new monoclonal OTP antibodies to allow application in clinical diagnostics. Finally, in **chapter 9**, we report the first patient-derived tumor organoids (PDTOs) from pulmonary neuroendocrine neoplasms, address the fidelity of the model, and uncover potential therapeutic vulnerabilities. In the general discussion provided in **Chapter 10**, we further elaborate on new insights to improve both the diagnostic and prognostic classification of pulmonary carcinoids. Finally, a summary of the dissertation is provided.

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CHAPTER 2

Disease relapse in relation to extent of lymph node sampling in patients with resected pulmonary carcinoid tumors: a population-based study

L. Moonen, J. L. Derks, L. M. Hillen, R. J. van Suylen,
M. A. den Bakker, J. H. von der Thüsen, R. A. Damhuis,
W. A. Buikhuisen, E. C. van den Broek, J. Maessen,
A. P. W. M. Maat, P. van Schil, E. J. M. Speel, A-M. C. Dingemans

Submitted

CHAPTER 3

Preoperative biopsy diagnosis in pulmonary carcinoids, a shot in the dark

L. Moonen, J. L. Derks, B. C. M. Hermans, I. M. Bunnik, L. M. Hillen, R. J. van Suylen, M. A. den Bakker, J. H. von der Thüsen, R. A. Damhuis, E. C. van den Broek, W. A. Buikhuisen, A-M. C. Dingemans, E. J. M. Speel

J Thorac Oncol 2021;16(4):610-618

Abstract

Introduction

The preferred treatment for pulmonary carcinoids (PCs) is lobectomy, and parenchyma-sparing approaches might be considered for typical carcinoids (TCs). Treatment decisions are based on a preoperative biopsy diagnosis. Following the WHO criteria (2015), definitive diagnosis is only feasible postoperatively, thereby hampering preoperative treatment decisions. Here, we determined whether the final carcinoid classification on a resection specimen can be predicted by a preoperative biopsy.

Methods

We searched all stage I to III patients with a final carcinoid diagnosis who underwent a curative resection and of whom both a preoperative biopsy and paired resection specimen were available (2003-2012) using the Dutch Pathology Registry (PALGA) and the Netherlands Cancer Registry (NCR). Pathology report conclusions of the biopsy-resection specimen were compared.

Results

Paired biopsy-resection specimens in combination with clinical data were available from 330 patients. 57% (189 of 330) of the patients exhibited discordance between the preoperative biopsy and paired resection diagnosis, including 36% (44 of 121) preoperatively diagnosed TC, 40% (6 of 15) atypical carcinoid (AC), and 65% (103 of 158) not-otherwise-specified (NOS) carcinoids. A quarter of preoperatively diagnosed TC and NOS were reclassified as AC on the resection specimen. Preoperatively diagnosed ACs exhibited the highest relapse rates (40%, 6 of 15). Preoperatively diagnosed TC and NOS patients who were reclassified as ACs exhibited higher relapse rates as compared to nonreclassified TCs and NOS (3% versus 1%, and 16% versus 6%).

Conclusions

We provide evidence that carcinoid classification on preoperative biopsies is imprecise, as is also stated by the current WHO classification. We advise clinicians to interpret the preoperative biopsy diagnosis with caution in deciding the extent of surgery (e.g., parenchyma-sparing versus non-parenchyma-sparing).

Introduction

Pulmonary carcinoids (PC) represent a subgroup of lung cancer that, together with large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC), are referred to as pulmonary neuroendocrine neoplasms (NENs).[1] Following the World Health Organization (WHO) 2015 classification, PCs can be classified into low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) on the basis of the mitotic count (TC 0-1 and AC ≥2-10 mitoses per $2mm^2$) and the presence of necrosis (AC)[1]. Although carcinoids have a relatively indolent behaviour, they may invade and metastasize. In general, ACs are characterized by a more aggressive disease course as compared with TCs because they more often exhibit local disease relapse and have a higher propensity to metastasize.[2] The preferred treatment for local-regional carcinoid disease is surgery.[2] Parenchyma-sparing strategies, such as sublobar or endobronchial resection, may be considered for TC, whereas lobectomy is advised for AC.[3-13] These treatment decisions are based on a pathological diagnosis obtained from a preoperative tumor biopsy. WHO 2015 criteria have provided new criteria for the diagnosis of lung cancer on the basis of small biopsies and cytology, notwithstanding criteria for PCs are still lacking. It is advised to use the Ki67 labeling index to avoid misdiagnosing carcinoid tumors as high-grade neuroendocrine tumors. However, the use of this marker to discriminate TC from AC or to predict prognosis within the individual carcinoid tumor categories is not yet established.[1,14] Therefore, definitive diagnosis of TC or AC is only feasible postoperatively, which hampers preoperative treatment decisions, on the basis of histologic subtypes. Even though PCs are often diagnosed on a biopsy specimen in current practice, the diagnostic accuracy of pulmonary biopsies has not been thoroughly investigated. Hence, we analyzed all carcinoid diagnoses established in the Netherlands between January 2003 and December 2012, using both the Dutch Pathology Registry (PALGA) and the Netherlands Cancer Registry (NCR), and we compared the postoperative diagnoses with the diagnoses determined on the preoperative biopsy specimens.

Methods

Selection of cases

All data for this study were retrospectively retrieved from PALGA[15], the nationwide network and registry of histopathologic and cytopathologic specimen in the Netherlands, and the NCR. The study protocol was approved by the medical ethical committee of Maastricht University Medical Centre (METC 16-4-106) and was performed according to the Dutch Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011) regulations not requiring patient informed consent. Written conclusions in

pathology reports describing PC, TC, or AC diagnosed between January 1, 2003 and December 31, 2012 were retrieved.

All nationwide available pathological diagnoses for each individual patient were gathered by searching PALGA from ≤15 years after and before the first carcinoid diagnosis updated until January 1, 2017. Subsequently, all pathology conclusions were screened by two researchers (I.B. and J.D.). Cases without a definitive PC diagnosis were excluded (such as high-grade neuroendocrine carcinoma, non-neuroendocrine tumor, neuroendocrine tumor of nonpulmonary origin). PALGA data were coupled to clinical data from the NCR, and only patients with stage I to III carcinoid who underwent a curative resection were selected. Subsequently, patients with both a pathology conclusion on the preoperative biopsy (transbronchial, endobronchial, or needle) and the resection specimen available were selected and included in this study (Figure 3.1).

Assessment of pathological diagnosis

Diagnoses based on the prevailing WHO criteria, without central pathological review, of both the biopsy specimen and resection specimen from the included patients were clustered into subgroups by applying the criteria listed in Table 3.1 (TC, AC, not-otherwise-specified (NOS) carcinoid, high-grade neuroendocrine carcinoma (HGNEC), and other). Patients with ‘carcinoid’ or ‘neuroendocrine tumor’ and ‘grade 1’ or ‘typical’ were included in the ‘typical carcinoid’ cluster. Patients with ‘carcinoid’ or ‘neuroendocrine tumor’ and ‘grade 2’ or ‘atypical’ were allocated to the ‘atypical carcinoid’ group. All other patients with ‘carcinoid’ or ‘neuroendocrine tumor’ but no or unclear further differentiation were grouped as ‘carcinoid NOS’. All cases with ‘neuroendocrine carcinoma (small cell/large cell)’, ‘high-grade’ or ‘poorly differentiated’ were allocated to the HGNEC group. All other cases were allocated to the other cluster (Table 3.1). In case a revision of the initial diagnosis was performed (i.e., a second opinion was provided), the revised diagnosis was used in the analysis.

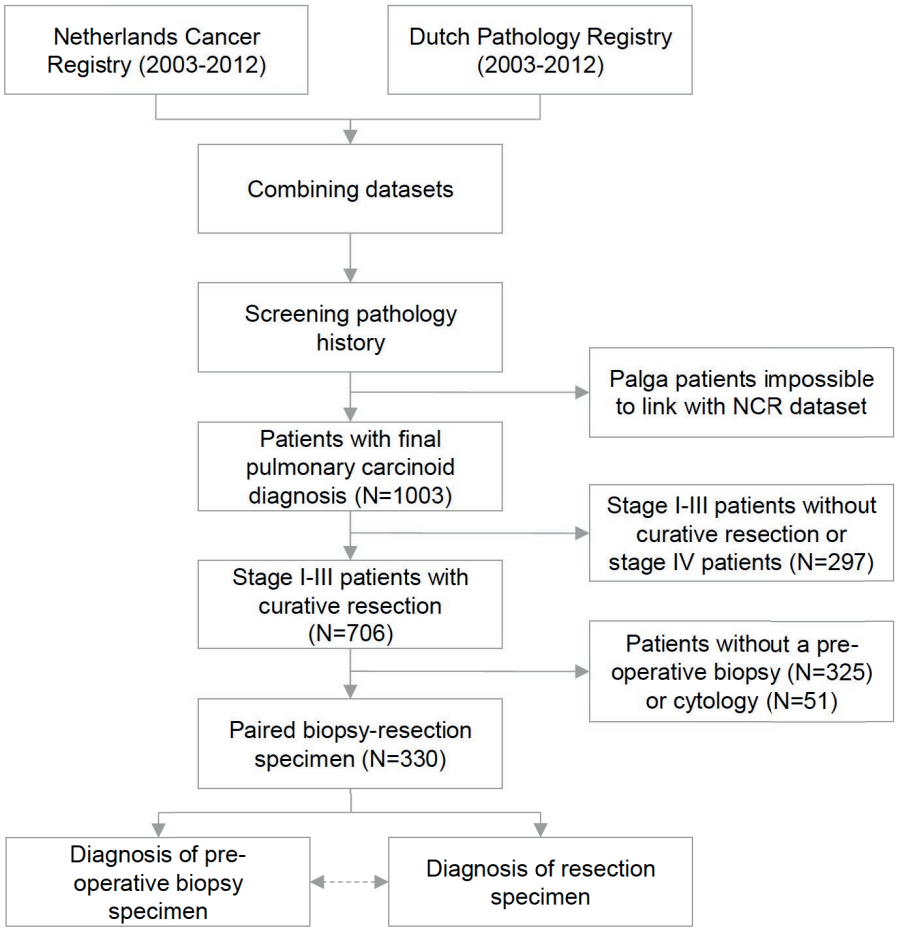

Figure 3.1 Flowchart presenting an overview of the selection procedure of conclusions from pathology reports leaving 330 combined biopsy resection specimens.

Pathological and clinical data

The following data were extracted from PALGA: tumor sampling location (e.g., lung, liver, lymph node), diagnosis recorded in the conclusion, the origin of the sample (e.g., primary tumor versus metastasis), location of the primary tumor, sampling method, time between biopsy and resection in days. After extracting information on the sampling method, cases were further subdivided into nonsurgically obtained biopsy specimens (endobronchial and needle) and resection specimens (any surgical resection).

Pathologically confirmed disease relapse was subtracted from the PALGA database. Relapse was defined as any diagnostic report mentioning a carcinoid tumor metastasis (local and distant). Data was last updated for all included patients on January 1, 2017.

Clinical data from NCR included the year of diagnosis, Tumor-Node-Metastasis (TNM) classification (≥2010 according to the TNM-7 and ≤2009 according to the TNM-6 classification), first-line treatment modality, time from biopsy specimen diagnosis to date of resection, disease relapse, and time from date of resection till the relapse date, date of last follow-up, date of second primary cancer with distant metastasis, or death. Clinical follow-up was updated, using the NCR database, until February 1, 2019.

Table 3.1 Nomenclature used to cluster carcinoid diagnoses retrieved from pathology conclusions.

	Words required for clustering		
Clustered diagnosis	Word 1 (any of the following)	And	Word 2 (any of the following)
Typical carcinoid	Carcinoid, neuroendocrine tumor		Typical, Grade I
Atypical carcinoid	Carcinoid, neuroendocrine tumor		Atypical, Grade II
Carcinoid NOS	Carcinoid, neuroendocrine tumor		Unsure if typical or atypical, well differentiated, low grade, not otherwise specified
HGNEC*	Neuroendocrine (large/non-small cell) Ca, Combined large/small cell Ca		High-grade, poorly differentiated, intermediate cell type
Other*	Ca (large/non-small cell), Adenocarcinoma, Squamous cell carcinoma, Unclear whether Ca or carcinoid, All conclusions with uncertainty (i.e., more than 2 possible differential diagnosis)		(IHC) neuroendocrine features, (IHC) neuroendocrine differentiation, endocrine features

* Additional clinical data of both the HGNEC and Other group are provided (see Table S3.1, Supplemental Data). Ca, carcinoma; HGNEC, High grade neuroendocrine carcinoma; IHC, Immunohistochemical; NOS, not otherwise specified.

Data analysis

The diagnosis of the preoperative biopsy was compared with the diagnosis established on the resection specimen of the paired biopsy and resection specimen for each patient. The resection diagnosis was regarded as the accepted standard. Percentages of correct diagnoses and percentages and nature of false diagnoses on preoperative biopsy are presented. Cases in which the resection diagnosis did not match the biopsy diagnosis were evaluated for the type of biopsy taken and the extent of surgery performed. In addition, we investigated if carcinoid relapse was related to the type of surgical resection. Relapse-free survival (RFS) was defined as the time from initial surgical treatment until disease relapse. Censoring for RFS occurred on the last day of follow-up, death (without evidence of disease based on NCR and PALGA database) or date of second primary cancer with distant metastases as from this moment onward, further assessment of carcinoid tumor progression was not possible anymore.

Statistical analysis

Data were analyzed using the descriptive statistics function of IBM SPSS software for Mac version 26 (SPSS, Inc., Chicago, IL, USA). The chi-squared test and Fisher's exact test were used to compare categorial data. RFS was estimated according to the Kaplan-Meier method and tested using the log-rank test. As the median RFS was not reached, we calculated the percentage of patients without disease relapse at 3,5, and 10 years after surgery. Kaplan-Meier was drawn using R package survminer (version 0.4.2). Two-sided *p* values <0.05 were considered significant.

Results

Selection of cases

After combining and screening of the PALGA and NCR database, 1003 unique patients with a final carcinoid diagnosis were retrieved (Figure 3.1). Paired biopsy and resection specimens were available for 330 patients. The final diagnosis, based on the resection specimen, was TC in 160 (48.5%) patients, AC in 88 (26.7%) patients, and carcinoid NOS in 82 (24.8%) patients. The median time between the biopsy and resection procedure was 41 days (interquartile range, 27-65 days). Most (94.5%) preoperative biopsy specimens were endobronchial biopsy specimen.

Preoperative biopsy diagnosis *versus* resection diagnosis

Preoperative biopsy diagnoses included TC (36.6%, n=121 of 330), AC (4.5%, n=15 of 330), carcinoid NOS (47.9%, n=158 of 330); furthermore, this cohort of 330 patients included 36 patients with a final carcinoid diagnosis who were preoperatively diagnosed as HGNEC (1.8%, n=6 of 330) and other (9.1%, n=30 of 330). In 57% (n=189 of 330) of the patients the preoperative biopsy specimen diagnosis did not match the paired final diagnosis. This was the case in 36% (n=44 of 121) of the patients with a preoperative TC diagnosis, 40% (n=6 of 15) with AC, and 65% (n=103 of 158) with carcinoid NOS, respectively (Figure 3.2a). In 24% (n=29 of 121) of patients with a preoperative TC diagnosis, the final diagnoses on the resection specimen revealed an AC (Figure 3.2a). In biopsy diagnosed AC, 40% (n=6 of 15) were eventually diagnosed as TC on the resection specimen (Figure 3.2a). From the patients with a preoperative diagnosis of carcinoid NOS, the resection specimen diagnoses showed TC in 41.8% (n=66 of 158) of the patients, AC in 23.4% (n=37 of 158), and 34.8% (n=55 of 158) remained carcinoid NOS, respectively (Figure 3.2a). All patients preoperatively diagnosed as HGNEC (n=6) or other NE tumor (n=30) were downgraded to TC (33% (n=2 of 6), 30% (n=9 of 30)), AC (67% (n=4

of 6), 30% (n=9 of 30)), and carcinoid NOS (40% (n=12 of 30)) on the resection specimen (Figure 3.2a).

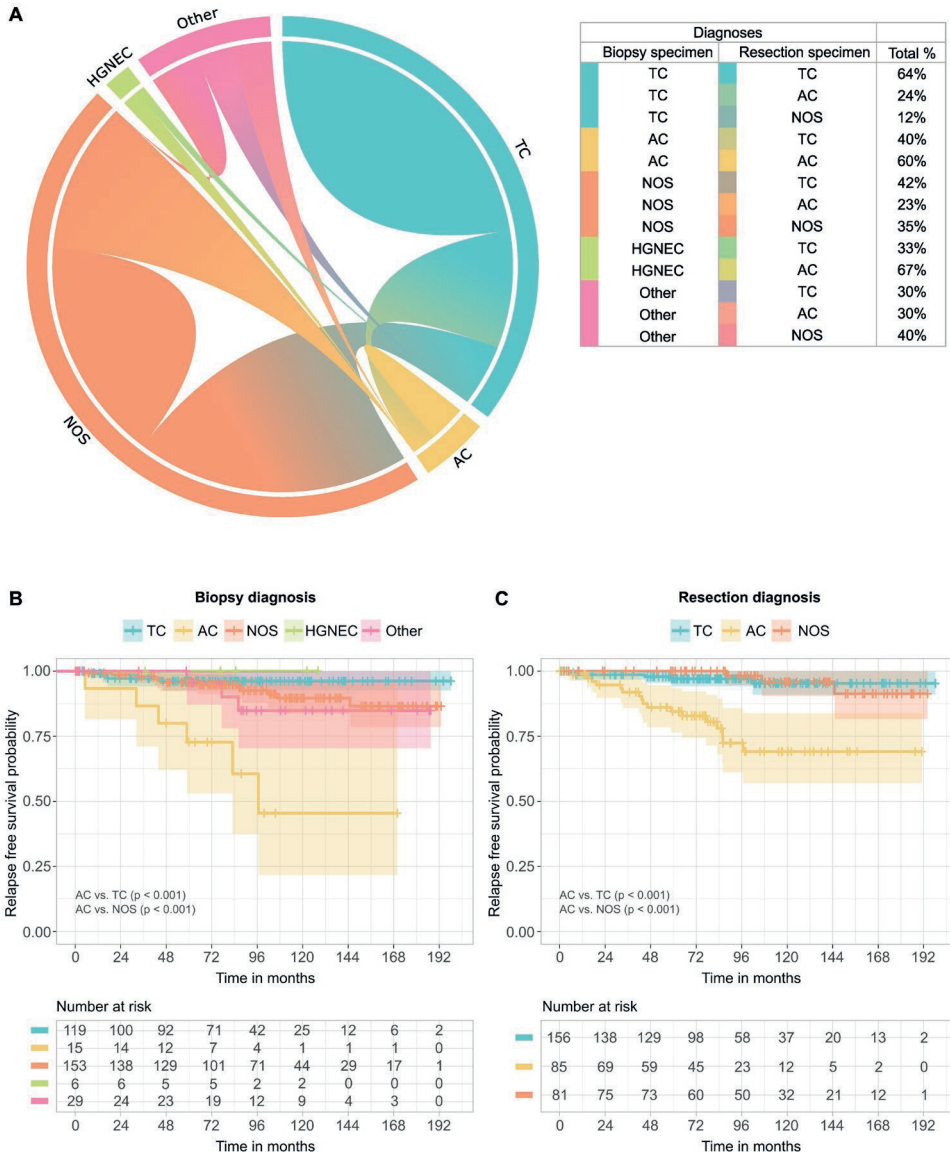

Figure 3.2 (**A**) Chord diagram illustrating concordance and discordance between the preoperative diagnosis (outer ring) and the corresponding resection specimen diagnosis (inner flows). (**B**) Biopsy specimen diagnosis; Relapse-free survival probability. (**C**) Resection specimen diagnosis; Relapse-free survival probability. Abbreviations: AC, atypical carcinoid; HGNEC, high-grade neuroendocrine carcinoma; NOS, not-otherwise-specified; TC, typical carcinoid.

Type of treatment

To investigate whether the preoperative biopsy diagnosis may have influenced the extent of surgical treatment, we evaluated the initial surgical treatment per type of preoperative biopsy diagnosis (e.g., TC, AC, carcinoid NOS, HGNEC, other). In 92% (n=304 of 330) of patients a (bi)lobectomy or pneumonectomy was performed (Table 3.2). Lobectomy represents a cluster of patients treated with a lobectomy (n=176 of 274), bilobectomy (n=60 of 274), and sleeve lobectomy (n=38 of 274) (Table 3.2). Wedge or segment resections were performed infrequently in 5% (n=15 of 330) of the patients, all with a preoperative non-AC diagnosis (TC (n=7) or carcinoid NOS (n=8)). In two of these cases, the final diagnosis was AC, whereas four remained carcinoid NOS. In the case of a preoperative AC, HGNEC, or other diagnosis, the treatment was lobectomy or pneumectomy (Table 3.2).

Table 3.2 Overview presenting the preoperative diagnosis with the combined postoperative diagnosis and the corresponding type of surgery performed.

Diagnosis		Type of surgery N (%)						
Pre-operative	Post-operative	Total	Wedge	Segment	Bisegment	Lobectomy*	Pneumectomy	Unknown
TC	TC	77 (100)	4 (5)	2 (3)	0 (0)	63 (82)	6 (8)	2 (3)
TC	AC	29 (100)	0 (0)	0 (0)	0 (0)	25 (86)	3 (10)	1 (3)
TC	NOS	15 (100)	0 (0)	1 (7)	0 (0)	13 (87)	1 (7)	0 (0)
AC	TC	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)	0 (0)	0 (0)
AC	AC	9 (100)	0 (0)	0 (0)	0 (0)	8 (89)	1 (11)	0 (0)
NOS	TC	66 (100)	1 (2)	2 (3)	0 (0)	52 (79)	9 (14)	2 (3)
NOS	AC	37 (100)	1 (3)	1 (3)	0 (0)	31 (84)	0 (0)	4 (11)
NOS	NOS	55 (100)	3 (5)	0 (0)	1 (2)	47 (85)	4 (7)	0 (0)
HGNEC	TC	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
HGNEC	AC	4 (100)	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)	0 (0)
Other	TC	9 (100)	0 (0)	0 (0)	0 (0)	7 (78)	2 (22)	0 (0)
Other	AC	9 (100)	0 (0)	0 (0)	0 (0)	8 (89)	1 (11)	0 (0)
Other	NOS	12 (100)	0 (0)	0 (0)	0 (0)	10 (83)	1 (8)	1 (8)
Total		330 (100)	9 (3)	6 (2)	1 (0)	274 (83)	30 (9)	10 (3)

*Lobectomy represents a cluster of lobectomy, bilobectomy, and sleeve lobectomy as surgical procedures. Abbreviations: AC, atypical carcinoid; HGNEC, high-grade neuroendocrine carcinoma; NOS, carcinoid not otherwise specified; TC, typical carcinoid.

Relapse of disease

The median follow-up was 88.6 months (95% confidence interval (CI), 82.0-95.3). During follow-up, 8% (25 of 330) of the patients revealed relapse of disease (Table 3.3). This was the case in 3% (n=5 of 160) of patients with TC, in 19% (n=17 of 88) of patients with AC, and in 4% (n=3 of 82) of carcinoid NOS patients, as assessed in the resection specimen (Table 3.3). 16% (n=4 of 25) of the patients revealed local relapse whereas 80% (n=20 of 25) revealed distant relapse. In addition, one patient showed both local and distant

relapse. All patients with preoperatively diagnosed AC who relapsed (n=6) revealed distant disease relapse, whereas local disease relapse was observed in preoperatively diagnosed TC (n=2 of 4), NOS (n=1 of 11), and other (n=1 of 4). The highest relapse rates were observed in preoperatively diagnosed AC patients (40%, n=6 of 15). Preoperatively TCs and NOS patients who were reclassified as ACs revealed higher relapse rates as compared with nonreclassified TCs and NOS (3% versus 1%, and 16% versus 6%, respectively). A total of 88% (22 of 25) of the relapses were observed in patients who underwent lobectomy (Table 3.3). Nevertheless, no association was observed between lobectomy and relapse of disease (*p*=0.550).

Table 3.3 Overview presenting the number of relapses based on the preoperative diagnosis stratified by type of treatment.

Diagnosis		Total cohort	Relapse cases per type of surgery N (%)			
Pre-operative	Post-operative	Total cases N	Lobectomy*	Pneumectomy	Unknown	Total relapses
TC	TC	77	1 (1)	0 (0)	0 (0)	1 (1)
TC	AC	29	1 (3)	1 (3)	1 (3)	3 (10)
TC	NOS	15	0 (0)	0 (0)	0 (0)	0 (0)
AC	TC	6	1 (17)	0 (0)	0 (0)	1 (17)
AC	AC	9	5 (56)	0 (0)	0 (0)	5 (56)
NOS	TC	66	2 (3)	1 (2)	0 (0)	3 (5)
NOS	AC	37	6 (16)	0 (0)	0 (0)	6 (16)
NOS	NOS	55	3 (5)	0 (0)	0 (0)	3 (5)
HGNEC	TC	2	0 (0)	0 (0)	0 (0)	0 (0)
HGNEC	AC	4	0 (0)	0 (0)	0 (0)	0 (0)
Other	TC	9	0 (0)	0 (0)	0 (0)	0 (0)
Other	AC	9	3 (33)	0 (0)	0 (0)	3 (33)
Other	NOS	12	0 (0)	0 (0)	0 (0)	0 (0)
Total		330	22 (7)	2 (1)	1 (0)	25 (8)

*Lobectomy represents a cluster of lobectomy, bilobectomy, and sleeve lobectomy as surgical procedures. Abbreviations: AC, atypical carcinoid; HGNEC, high-grade neuroendocrine carcinoma; NOS, carcinoid not otherwise specified; TC, typical carcinoid.

Relapse free survival

Data on RFS were available in 322 patients. A total of 25 patients progressed, and 55 patients died during the study period. An overview of the RFS is provided in Table 3.4. Patients preoperatively diagnosed with AC revealed a significantly worse RFS compared with patients preoperatively diagnosed with non-AC (e.g., TC and NOS) diagnosed patients (*p*=2,02E-7) (Table 3.4, Figure 3.2b). Patients postoperatively diagnosed with TC and NOS revealed no significant difference in RFS (*p*=0.821), whereas patients postoperatively diagnosed with AC revealed a significantly worse RFS as compared to TC (*p*=0,3E-6) and NOS (*p*=3,7E-5) patients (Figure 3.2c).

Table 3.4 Overview presenting the percentage 3, 5, and 10-year RFS stratified by the preoperative and postoperative diagnosis.

	Preoperative diagnosis (N)					Postoperative diagnosis (N)		
RFS	TC (119)	AC (15)	NOS (153)	HGNEC (6)	Other (29)	TC (156)	AC (85)	NOS (81)
3-year	97.5%	86.7%	98.0%	100%	100%	98.7%	92.9%	100%
5-year	96.6%	73.3%	96.1%	100%	96.6%	97.4%	87.1%	100%
10-year	96.6%	60.0%	92.8%	100%	89.7%	96.8%	80.0%	97.5%

Abbreviations: AC, atypical carcinoid; HGNEC, high-grade neuroendocrine carcinoma; RFS, relapse-free survival; NOS, carcinoid not otherwise specified; TC, typical carcinoid.

Discussion

The WHO 2015 classification of pulmonary NEN discourages a diagnosis of TC or AC on a biopsy specimen. However, until now, this advice has not been supported by solid clinical data. In this study, we found that, in daily clinical practice, in approximately half of the carcinoid patients (i.e., 57%), a discordance exists between the diagnosis obtained on a preoperative biopsy specimen and the corresponding resection specimen diagnosis. Up to 24% of preoperatively diagnosed (typical) carcinoids were revised to AC on the resection specimen and these patients showed higher relapse rates. These data confirm that the classification of PC tumors on biopsy specimens is challenging in daily practice and that one should be cautious in deciding the extent of surgical resection (e.g., parenchyma-sparing versus lobectomy) based on a preoperatively established carcinoid diagnosis.

Carcinoid diagnosis (i.e., TC versus AC) is currently based on the mitotic index and the presence of necrosis. The assessment of these morphological features can already be challenging on correctly handled and optimally fixed surgical resection specimens, which makes it considerably harder on small biopsy specimens.[16] In addition, inadequate handling, fixation, and processing have more impact on biopsies than resection specimens and may lead to morphologic changes such as nuclear chromatin condensation and shrinking, thereby increasing the risk of preoperative diagnostic misinterpretations.[17] Indeed, other technical issues such as the relatively large microscopic area containing a rather small number of mitoses, the disparate distribution of mitoses within the tissue section, the fact that a mitotic figure may be misinterpreted as an apoptotic cell, a crushed cell, or a granulocyte nucleus, and the heterogeneity of pulmonary tumors, may also be of influence.[18] Overall, it has to be questioned whether a small biopsy specimen is a representative reflection of the tumor and whether it is reliable in preoperative PC diagnosis given the consequences it may entail.
In addition to technical issues, we investigated whether the type of preoperative biopsy (e.g., endobronchial, needle, or excision) was of influence on the diagnostic discordance.

However, as our results revealed that most preoperative biopsy specimens were endobronchial biopsies as compared to excision and needle biopsies (95% versus 1% and 4%), no conclusions could be drawn on the influence of the type of biopsy on the correctness of the preoperative diagnosis.

Although the preferred treatment for carcinoid tumors, whether TC or AC, is anatomical lobectomy, several recent retrospective studies have reported the noninferiority of parenchyma-sparing resections (e.g., wedge resection and segmentectomy) compared with the traditionally advised lobectomy for TC.[6,8,10,12,13] Based on these studies, the European Neuroendocrine Tumor Society (ENETS) guidelines propose to consider segmental resection (rather than wedge resection) in patients with limited pulmonary function[2]. Furthermore, ENETs recommends performing parenchyma-sparing surgery for patients with central airway tumors. These tumors are almost exclusively TC and are, thus, characterized by a low recurrence potential.[2] However, the National Comprehensive Cancer Network (NCCN) guidelines still advise lobectomy or another anatomical resection for TC, and the European Society for Medical Oncology (ESMO) guidelines prefer lobectomy.[19,20] Furthermore, other studies have suggested endobronchial treatment for centrally-located TC with high success rates and limited recurrences.[3-5,9,11] None of the guidelines advise parenchyma-sparing resection for AC, which is in line with our data illustrating that no parenchyma-sparing strategies were applied to AC. Indeed, the frequent relapse of disease in post-operatively diagnosed AC (19%, n=17 of 88) underscores that traditional oncologic anatomical surgery is required in these patients. These findings are also supported by a study of Filosso *et al.* (n=126) reporting a sublobar resection (e.g., wedge resection or segmentectomy) as an independent negative prognostic factor for AC.[7] In addition, it needs to be emphasized that a sublobar resection requires a dedicated lymph node dissection to minimize the risk of incomplete resection.

Thus, the different treatment options for TC and AC, and the limited concordance between the preoperative and postoperative diagnosis emphasize a serious clinical dilemma when it comes to treatment management based on the basis of a preoperative biopsy diagnosis. We did not observe a difference in patient outcomes with regard to disease relapse. However, we did observe a significantly lower RFS for patients diagnosed with AC as compared to both TC (p=0.3E-6) and NOS (p=3.7E-5).

Accurate histopathological diagnosis and prediction of malignancy of lung NET biopsies may require additional analyses besides assessment of morphologic characteristics. A potential marker of additive value is the Ki67 immunohistochemical proliferation index, also known as Mib-1. Evaluation of manual counting of Ki67 stained lung NET biopsies

and their paired resection specimens revealed a strong correlation, independent of tumor type, biopsy size, tumor sampling method, and heterogeneity in the distribution.[21] Others have already reported the diagnostic use of Ki67 to separate low-grade NETs (i.e., TC and AC) from small cell carcinoma.[17,21] Furthermore, the Ki67 labelling index has potential diagnostic, prognostic, and grading implications in surgically resected lung NETs.[22] However, in the current literature, cut off values for Ki67 proliferation index to subclassify carcinoids are contradictory and therefore not yet advised by the WHO classification.

Additional immunohistochemical markers, known to be able to predict a favorable prognosis in PCs, are the nuclear expression of Orthopedia Homeobox (OTP) and membranous expression of CD44.[16,23-26] The combination of OTP and CD44 has recently been proposed to identify patients at risk for disease relapse after surgery.[24] Another marker that correlates with prognosis is multiple endocrine neoplasia type 1 (MEN1). Several studies have reported that MEN1 mutation, which correlates with low gene expression, is related to poor prognosis in carcinoids.[27-30] Hence it is tempting to speculate that a panel of multiple (immunohistochemical) markers (Ki67, OTP, CD44, and MEN1) in addition to the current morphologic WHO classification may be used in future clinical practice to preoperatively identify patients who are at (very) low risk for disease relapse. For these patients, parenchyma-sparing surgery and a less intense follow-up period could be considered, especially in patients who have high cardiovascular comorbidity or a limited lung capacity.

This study has several limitations. First, it is a retrospective study relying on pathology report conclusions of diagnoses that have not been additionally confirmed by other pathologists. This reflects daily practice in which not all diagnoses are double-read by other pathologists, yet are used by clinicians to determine treatment. Second, our data revealed that a large proportion (35%, n=55 of 158) of the preoperatively diagnosed carcinoid NOS remained carcinoid NOS on the paired resection specimen. This diagnosis is insufficient because subclassification into TC versus AC is essential for, amongst others, treatment decisions and prognosis.[31] Nevertheless, these carcinoid NOS cases likely represent TC as their RFS was similar. Third, our data revealed that only a small proportion of the patients (5%, n=15 of 330) underwent parenchyma-sparing surgery. This might be owing to the timeframe of our cohort (2003-2012) in which limited literature regarding parenchyma-sparing strategies was available. It might be possible that parenchyma-sparing strategies are currently more frequently performed. This is confirmed in a recent study of Cattoni *et al*. analyzing 510 patients who underwent lung resection for a primary NET between 2000 and 2015. Results revealed that 22% (n=110 of 510) of the patients underwent either a wedge (n=77) or segmentectomy (n=33).[32]

The wedge resections were performed in 59 patients with TC, 9 patients with AC, and 9 patients with LCNEC.

Conclusion

Our data reveal that carcinoid diagnosis on a histologic biopsy specimen is imprecise; in half of the patients with a carcinoid tumor, the preoperative diagnosis does not match the accepted standard resection specimen diagnosis in current clinical practice. More important, this frequently includes 'upgrading' from preoperative non-AC to a postoperative AC diagnosis. We advise clinicians to interpret the preoperative biopsy diagnosis with caution in deciding the extent of surgery (e.g., parenchyma-sparing versus non-parenchyma-sparing). Future studies to improve the diagnostic and prognostic accuracy of preoperative PC biopsy specimens are urgently required.

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Supplemental material

Table S3.1 Overview of clinical data of the HGNEC and Other group.

Cluster	Preoperative diagnosis	Postoperative diagnosis	Gender	Age	Surgery	Relapse	Vital status	RFS (months)
HGNEC	LCNEC	AC	M	70	Lobectomy	No	dead	36.8
HGNEC	LCNEC	AC	M	73	Pneumonectomy	No	dead	76.7
HGNEC	LCNEC	AC	F	40	Lobectomy	No	alive	76.6
HGNEC	SCLC	AC	F	54	Pneumonectomy	No	alive	122.2
HGNEC	SCLC	TC	M	45	Lobectomy	No	alive	128.1
HGNEC	SCLC	AC	F	43	Lobectomy	No	alive	84.6
Other	Large cell carcinoma	AC	M	76	Lobectomy	No	Alive	187.5
Other	Adenocarcinoma	AC	M	60	Lobectomy	Yes	Dead	58.8
Other	Large cell carcinoma	NOS	M	70	Lobectomy	No	Alive	125.1
Other	NE carcinoma or carcinoid	NOS	F	22	unknown	No	Alive	n/a
Other	NE tumor; SCLC or AC	NOS	M	46	Lobectomy	No	Alive	2.3
Other	SqCC	NOS	F	67	Lobectomy	No	Alive	186.7
Other	NE carcinoma; LCNEC or AC	NOS	M	64	Bilobectomy	No	Alive	141.2
Other	NE tumor dd carcinoid or SCLC	NOS	M	60	Bilobectomy	No	Dead	166.8
Other	Adenocarcinoma	AC	M	34	Lobectomy	Yes	Alive	86.1
Other	Adenocarcinoma with NE features	NOS	F	65	Lobectomy	No	Alive	180.4
Other	(Atypical) carcinoid or NE carcinoma	NOS	M	73	Lobectomy	No	Dead	108.4
Other	Adenocarcinoma or carcinoid	TC	F	42	sleeve lobectomy	No	Alive	75.9
Other	(Atypical) carcinoid or NE carcinoma	AC	F	70	Bilobectomy	Yes	Dead	77.2
Other	NSCLC (NE features) or NE carcinoma	TC	M	59	Pneumonectomy	No	Alive	135.3
Other	NE tumor; SCLC or AC	AC	F	59	Lobectomy	No	Alive	132.1
Other	Adenocarcinoma	TC	M	59	Pneumonectomy	No	Dead	0.2
Other	Non-small cel carcinoma NE features	TC	F	66	Lobectomy	No	Alive	62.0
Other	Adenocarcinoma	NOS	F	59	Lobectomy	No	Dead	4.7
Other	Adenocarcinoma (NE differentiation)	AC	F	55	Pneumonectomy	No	Alive	90.4
Other	Adenocarcinoma	TC	M	63	Bilobectomy	No	Alive	33.5
Other	Adenocarcinoma	NOS	F	64	Lobectomy	No	Alive	121.5
Other	NE carcinoma; LCNEC or AC	AC	F	56	Lobectomy	No	Dead	1.7
Other	NE tumor; LCNEC or AC	NOS	F	58	Pneumonectomy	No	Alive	110.8
Other	Adenocarcinoma	TC	F	55	Lobectomy	No	Alive	95.5
Other	Adenocarcinoma (NE features) or LCNEC	AC	F	70	Lobectomy	No	Dead	2.1
Other	Adenocarcinoma or carcinoid	AC	F	57	Bilobectomy	No	Alive	98.1
Other	Neuroendocrine tumor NOS	TC	F	38	Lobectomy	No	Alive	87.6
Other	NE carcinoma; LCNEC or AC	TC	M	51	Lobectomy	No	Alive	61.5
Other	Adenocarcinoma	NOS	F	55	Lobectomy	No	Alive	58.6
Other	Adenocarcinoma	TC	F	58	Lobectomy	No	Alive	88.7

Abbreviations: RFS, relapse free survival; HGNEC, high grade neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma; AC, atypical carcinoid; M, male; F, female; SCLC, small cell lung cancer; TC, typical carcinoid; NOS, carcinoid not otherwise specified; NE, neuroendocrine.

CHAPTER 4

Preoperative biopsy diagnosis in patients with pulmonary carcinoids: a biomarker panel will be crucial to hit a bull's eye

L. Moonen, J. L. Derks, A-M. C. Dingemans, E. J. M. Speel
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To the editor

With interest, we have read the letter of Pelosi *et al*. entitled "Labelling lung neuroendocrine neoplasms for Ki-67 antigen to score a bull's-eye, not shoot in the dark'' which we received in response to our article on the accuracy of the preoperative biopsy diagnosis in patients with pulmonary carcinoids.[1] We would like to thank the authors for suggesting a practical solution to the shortcoming of the current morphology-based classification on preoperative biopsies (i.e., mitotic index and the presence of necrosis).

We are pleased to read that Pelosi *et al*.[2] underscores our concerns regarding the current histopathological classification on biopsy specimens and the clinical consequences it may entail. In our article, we quoted that accurate histopathological diagnosis and prediction of malignancy of lung neuroendocrine neoplasm biopsies may require additional molecular analysis, for example, assessment of the cell proliferation index (PI) using Ki-67 expression. Ki-67 results have not been reported in our study owing to inconsistent coverage of PI in the pathology reports. We commend the work performed by Pelosi *et al.* and agree with the potential additive value of PI to improve diagnostic agreement and thereby preoperatively treatment decision-making.[3,4] However, it needs to be emphasized that Ki-67 immunostaining is heterogeneous, containing hotspots in pulmonary carcinoids that might remain unsampled in a biopsy specimen resulting in overestimation or underestimation of grade. While several studies have revealed that a Ki-67 PI cut-off greater than 5% is associated with increased risk of distant metastasis[5-7], we and others revealed the limited prognostic value of Ki-67 in addition to the WHO classification[8, 9]. Because of the existence of overlap among Ki-67 cut-off values separating typical from atypical carcinoids, the Ki-67 PI is still not included in the WHO 2021 as a diagnostic criterion for pulmonary carcinoids.[10,11] Nevertheless, we are aware that Ki-67 is already frequently used in the current diagnostic workup by pathologists, as both European Neuroendocrine Tumor Society and North American Neuroendocrine Tumor Society guidelines advise to always include a Ki-67 PI in both surgical and biopsy specimens.

In our article, we propose OTP, CD44, and MEN1 as potential additional prognostic immunohistochemical markers. We agree with Pelosi *et al*. that, so far, data of these markers on biopsy specimen are limited. However, an increasing number of studies have investigated both the diagnostic and prognostic value of these markers exhibiting promising results on resection specimens.[5,12-18] We expect that the homogeneous staining pattern of OTP, and CD44 to a lesser extent, make these markers more reliable to interpret on preoperative biopsies as compared to Ki-67 alone. Moreover, all three markers are mentioned in the current WHO 2021 criteria as promising molecular

markers for the prognostication of pulmonary carcinoids.[10] In our opinion, this will, in time, result in more awareness among both pathologists and clinicians.

In summary, we underscore the need for additional preoperative tools that aid in both diagnosis and prognosis of pulmonary carcinoids. We feel that a panel of molecular markers (e.g., Ki-67, OTP, CD44, MEN1) applicable on preoperative biopsies will improve the diagnostic and prognostic accuracy of pulmonary carcinoids and may increase the chance to score a bulls-eye. Future studies should investigate these aspects comparing preoperative biopsies and paired resection specimens.

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CHAPTER 5

Orthopedia homeobox (OTP) in pulmonary neuroendocrine tumors: the diagnostic value and possible molecular interactions

L. Moonen, J. L. Derks, A-M. C. Dingemans, E. J. M. Speel
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Abstract

Generally, patients with stage I-IIIa (TNM) pulmonary carcinoid disease have a favourable prognosis after curative resection. Yet, distant recurrence of disease after curative surgery occurs in approximately 1–6% of patients with typical carcinoid and 14–29% in patients with atypical carcinoid disease, respectively. Known predictors of distant recurrence of disease are atypical carcinoid, lymphatic involvement, and incomplete resection status. However, none of them can be reliably used, alone or in combination, to exclude patients from long-term follow-up (advised 15 years). By genomic profiling, Orthopedia homeobox (*OTP*) has been identified as a promising prognostic marker for pulmonary carcinoid with a favourable prognosis and low risk of distant disease recurrence. Moreover, OTP is a highly specific marker for carcinoids of pulmonary origin and recent genome wide analysis has identified *OTP* as a crucial predictor of aggressive tumor behaviour. OTP in combination with CD44, a stem cell marker and cell-surface protein, enables the identification of patients with surgical resected carcinoid disease that could potentially be excluded from long-term follow-up. In future clinical practice OTP may enable clinicians to reduce the diagnostic burden and related distress and reduce costs of long-term radiological assessments in patients with a pulmonary carcinoid. This review addresses the current clinical value of OTP and the possible molecular mechanisms regulating OTP expression and function in pulmonary carcinoids.

Introduction

Pulmonary carcinoids (PC) are rare, well-differentiated neuroendocrine tumors accounting for 1-2% of all lung cancers.[1] Nevertheless, its occurrence has increased significantly over the past decades (approximately 6% per year over the past 30 years in both men and women).[2,3] In contrast to high-grade neuroendocrine lung carcinomas, such as large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC), carcinoids are characterized by a lower metastatic rate and a relatively favourable prognosis. According to the World Health Organization (WHO), PC are recognized morphologically by a neuroendocrine growth pattern and can be subdivided into typical carcinoids (TC) and atypical carcinoids (AC) based on the mitotic rate (less than 2/mm^2 for TC and between 2 to 10 for AC) and the presence of necrosis in ACs.[4] Although TCs and ACs are considered low- or intermediate-grade tumors, they may spread to regional lymph nodes and distant organs. TCs are characterized by a relatively favourable prognosis (5-20% metastasize), whilst ACs are more often characterized by a malignant behaviour and a lower 5- and 10-year survival rate (30-40% metastasize).[3] Although curative treatment by means of surgical resection is possible for most carcinoids, distant disease recurrence may still occur even up to 20 years after curative treatment.[1,5] More precisely, distant recurrence of disease after curative surgery ranges from 1-6% for TCs and 14-29% for ACs.[6-10] In current practice, recurrence in these patients is not predictable due to a lack of clinical-pathological features that enable accurate prediction of disease recurrence. As a consequence, all patients with PC require radiological assessment and follow-up for an extensive period of time (15-20 years).[1]

The requirement of surgical tissue for assessment of all diagnostic morphological criteria for PCs limits accurate diagnosis on small biopsies and cytology specimen. Besides histological classification, recent literature described the Ki-67 proliferation index as a valuable marker to distinguish carcinoid tumors (<20%) from high grade LCNEC and SCLC (≥20%) on biopsy specimens.[3,4,11,12] However, the utility of this marker to differentiate TCs from ACs or to predict prognosis within individual carcinoid tumors is limited.[13] These diagnostic limitations indicate the need for alternative molecular markers to subdivide carcinoids into clinical relevant categories.[4]

By expression profiling, Orthopedia Homeobox (*OTP*) has recently been identified as a reliable molecular marker to predict the prognosis of PC patients.[14] Although *OTP* has frequently been described as a key player in the development of the hypothalamic neuroendocrine system of vertebrates, its function in lung carcinoids remains to be elucidated. Here, we comprehensively review current literature on OTP function, OTP

expression in lung neuroendocrine neoplasms, and possible molecular pathways through which it might operate in both normal and pulmonary neuroendocrine tumor tissue.

OTP in relation to prognosis in PC

Though the role of OTP in pulmonary carcinoids is poorly understood, it has been described as a strong prognostic marker for pulmonary carcinoids.[14,15] In 2013, Swarts *et al.*, reported the molecular characterization of carcinoids in patients with prolonged and poor survival rates (n=10 discovery, n=54 validation).[12,14] Results revealed a set of downregulated genes in the unfavourable group, one of them showed a remarkably strong downregulation namely *OTP* (median fold change of 845).[16] Multivariate analysis, comparing *OTP* with clinical parameters, showed that loss of or decreased expression of *OTP* was independently associated with unfavourable survival and increased risk of metastases. These findings were validated at the protein level by immunohistochemistry using the rabbit-anti-OTP polyclonal antibody (clone HPA039365, Atlas Antibodies, Stockholm, Sweden).[14] The prognostic value of OTP has since been validated in larger series (n=288), confirming that loss of expression is associated with poor prognosis (Tables 5.1 and 5.2).[15,17]

Three different OTP expression patterns can be observed and are strongly related to patient outcome namely, a strong nuclear staining (nOTP) with or without cytoplasmic reactivity, an exclusively cytoplasmic staining (cOTP), and a negative staining pattern (Figure 6.1A-C).[14] Patients with nOTP expression have a favourable disease outcome, patients with cOTP reactivity have intermediate survival, and patients with absence of OTP expression rendered the worst disease outcome.[14] In addition, Swarts *et al.*, showed a strong correlation between absence of nOTP and the occurrence of distant metastasis (p=0.00014).[14] Most interestingly, we observed that OTP in combination with CD44, a cell-surface glycoprotein involved in cell-cell interactions, allowed for even better separation of tumors into prognostic relevant categories. These results have been independently confirmed by Papaxoinis *et al.*, evaluating 86 cases.[15] Results showed that CD44 and nOTP staining were an independent predictor for relapse-free survival (RFS) in patients with radically operated pulmonary carcinoids (Hazard Ratio (HR) 0.192, 95% Confidence Interval (CI) 0.064-0.574; p=0.03).[15] No statistically significant differences in RFS were observed for patients with tumors containing decreased expression of one or both proteins (p=0.861). For this reason, Papaxoinis *et al.*, proposed that a combination of CD44 and nOTP staining may be a prognostic marker to predict prognosis and development of recurrence of disease. To date, no evidence for a molecular interaction between OTP and CD44 has been reported.

Table 5.1 Overview of the characteristics of studies that analysed OTP expression in pulmonary carcinoids using immunohistochemistry.

Study, Year [Ref]	Study Population				Histology				WHO Classification (year)	Normal Tissue Included (*n*)	Other (NE) Tissues Included (*n*)
	Initial Cohort	Included in Analysis	Age (years)	Gender (*n*)	DIPNECH	TC	AC	HGNECs			
Hanley et al., 2018[18]	63	59	26–91	M(24), F(39)	0	9	6	1	Yes (2015)	No	Yes (51)
Nonaka et al., 2016*[17]	159	159	21–83	M(62), F(97)	7	123	21	104	Yes (2015)	Yes (*n*/a)	Yes (758)
Papaxoinis et al., 2018*[19]	166	166	16–83	M(62), F(104)	16	132	34	0	Yes (2015)	No	No
Papaxoinis et al., 2017*[15]	108	86	21–83	M(44), F(64)	8	69	17	0	Yes (2015)	No	No
Swarts et al., 2013[14]	352	348	16–83	M(130), F(159)	0	225	63	59	Yes (2003)	Yes (4)	Yes (9)
Yoxtheimer et al., 2018[20]	50	50	21–87	M(30), F(20)	0	8	6	16	Yes (2015)	No	Yes (20)
Viswanathan et al., 2019[21]	60	57	32–86	M(31), F(29)	0	11	12	19	Yes (2015)	No	Yes (18)

* studies performed in the same study population. Abbreviations: Ref, reference; n, number; M, male; F, female; DIPNECH, diffuse idiopathic neuroendocrine cell hyperplasia; TC, typical carcinoid; AC, atypical carcinoid; HGNECs, high-grade neuroendocrine carcinomas e.g., large cell neuroendocrine carcinoma and small cell lung carcinoma; WHO, world health organization; NE, neuroendocrine; IHC, immunohistochemistry; n/a, not applicable.

Table 5.2 Overview of immunohistochemical features of studies that performed OTP analyses.

Study Year [Ref]	Immunohistochemistry			Outcome (*n* OTP positive/total (%))			Staining Scoring	
	Antibody supplier#	Dilution	DIPNECH	TC	AC	HGNECs	Considered positive if	Overall conclusion
Hanley et al., 2018[18]	Sigma	(1:800)	-	9/9 (100%)	1/6 (17%)	-	Any percentage or intensity of nuclear OTP expression	OTP is a highly sensitive and specific marker for lung carcinoids
Nonaka et al., 2016*[17]	Atlas	(1:150)	7/7 (100%)	105/123 (85.4%)	10/21 (47.6%)	2/104 (1.9%)	1 + (1–25%), 2 + (25–50%), 3 + (50–75%), 4 + (>75%)	OTP may serve as a useful diagnostic marker for lung carcinoid tumors
Papaxoinis et al., 2018*[19]	Atlas	(1:150)	16/16 (100%)	117/132 (88.6%)	21/34 (61.8%)	-	More than 5% of the tumor expressed a positive reaction	OTP and TTF1 expression can be used to classify carcinoids into different clusters
Papaxoinis et al., 2017*[15]	Atlas	(1:150)	-	nOTP < 150 14/69 (20.3%) cOTP < 150 59/69 (86%) nOTP > 150 55/69 (80%) cOTP > 150 10/69 (14.5%)	nOTP <150 8/17 (47%) cOTP <150 14/17 (82%) nOTP > 150 9/17 (53%) cOTP > 150 3/17 (18%)	- - - -	H-score (ranging from 0–300)	CD44/nOTP expression is an independent predictor of RFS in patients with radically operated PCs
Swarts et al., 2013[14]	Atlas	(1:800)	-	nOTP 10/225 (4%) nOTP + cOTP 165/225 (73%) cOTP 17/225 (8%)	nOTP 3/63 (5%) nOTP + cOTP 28/63 (44%) cOTP 15/63 (24%)	nOTP 1/59 (2%) nOTP + cOTP 4/59 (7%) cOTP 8/59 (14%)	0=no staining, 1=very weak diffuse staining [cytoplasm] or staining in single or very few nuclei, 2=weak to moderate staining, for nuclear staining in >40% of nuclei, 3–4=strong to very strong staining in most or all tumor cells, respectively.	OTP and CD44 are powerful prognostic markers for pulmonary carcinoids
Yoxtheimer et al., 2018[20]	Sigma	(1:800)	-	4/8 (50%)	1/6 (17%)	1/16 (6.3%)	Min. 5% of tumor cell positivity of 3 + staining intensity	OTP may be used to grade pulmonary NETs and differentiate them from low-grade NETs originating in other sites
Viswanathan et al., 2019[21]	Sigma	(1:800)	-	9/11 (82%)	8/12 (80%)	0/19 (0%)	Tumor showed >1 + OTP staining in >5% of the tumor within the specimen	OTP is a promising highly sensitive and specific marker for primary pulmonary carcinoid tumors

* studies performed in the same study population. # All studies used the rabbit anti-OTP polyclonal antibody clone HPA039365. Abbreviations: Ref, reference; n, number; OTP, orthopedia homeobox; DIPNECH, diffuse idiopathic neuroendocrine cell hyperplasia; TC, typical carcinoid ;AC, atypical carcinoid; HGNECs, high-grade neuroendocrine carcinomas e.g. large cell neuroendocrine carcinoma and small cell lung carcinoma; TTF1, thyroid transcription factor 1; CD44, cell-surface glycoprotein; nOTP, nuclear OTP expression; cOTP, cytoplasmic OTP expression; RFS, Relapse free survival; PCs, pulmonary carcinoids ;NETs, neuroendocrine tumors.

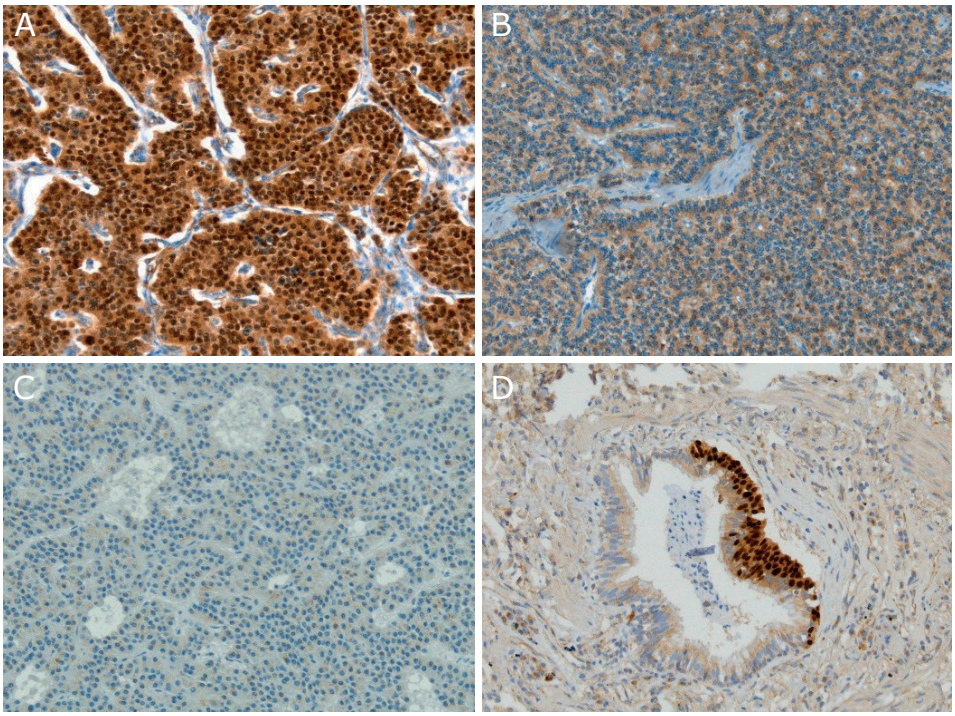

Figure 5.1 Orthopedia homeobox (OTP) immunohistochemistry of pulmonary carcinoids and neuroendocrine cell hyperplasia (NECH). (**A**) Representative image of nuclear OTP (nOTP) and cytoplasmic OTP (cOTP) staining in a typical carcinoid; (**B**) Representative image of a carcinoid tumor harbouring only cytoplasmic immunoreactivity for OTP; (**C**) Representative image of a carcinoid tumor with no OTP immunoreactivity; (**D**) Representative image of both nOTP and cOTP staining in NECH (magnification 200×)

Current studies indicate that protein expression of OTP/CD44, mainly nuclear staining, can be used to stratify PCs into prognostic relevant subgroups independent of the morphological established diagnosis.[14,15] In addition, due to the morphological similarities, the existing histology-based grading is problematic and subject to considerable interobserver variation. Swarts *et al.*, examined the interobserver variation among five experienced pulmonary pathologists who reviewed n=123 originally diagnosed pulmonary carcinoid cases.[22] In total 114 of the 123 cases were unanimously classified as pulmonary carcinoids. Fifty-five percent (n=63/114) were unanimously classified, 25% (n=29/114) reached consensus classification, and no consensus was reached for 19% (n=22/114), which comprised predominately ACs.[22] Although, consensus reclassification may improve prediction of survival of pulmonary NETs, it did not improve the prediction of prognosis of the disagreement cases (from p=0.11 to p=0.14).[22] Nevertheless, when disagreement cases were allocated on the basis of nOTP immunostaining patient

prognosis prediction improved significantly (from p=0.11 to p=0.0024). Thus, molecular markers may be used to classify carcinoids into prognostic relevant categories. Future studies should evaluate the diagnostic sensitivity and specificity of OTP as a marker to predict metastatic disease after curative surgery and if this marker is applicable for diagnostic and prognostic stratification on biopsy specimens.

OTP is specifically expressed within pulmonary carcinoids

Whilst OTP has been described as a prognostic marker for PCs, little is known about the expression pattern of this transcription factor in normal organs/tissues and other tumors. OTP expression in other neuroendocrine tumors and/or normal tissue and organs has been investigated in six studies.[14,17,18,20,21,23] Swarts *et al*., previously analysed the expression of *OTP* by qRT-PCR on frozen material of carcinoids, LCNEC, SCLC, normal tissues, and neuroendocrine cell lines (Bon-1, CM, NCI-H69, NCI-H295, NCI-H460, NCI-H720, NCI-H727, QGP and SW13). Carcinoid tumors showed a positive *OTP* messenger-RNA (mRNA) expression whereas normal tissues, high-grade neuroendocrine carcinomas, and the evaluated NE cell lines did not express *OTP* mRNA.[14] In addition, Nonaka *et al*., investigated immunohistochemical OTP expression in a variety of tumors, with special interest in pulmonary and non-pulmonary neuroendocrine tumors, neuroendocrine carcinomas, and normal tissues and organs.[17] Nuclear OTP expression was observed in 80% (n=130/162) of all pulmonary carcinoid tumors. Four out of 34 small cell carcinomas showed focal expression of OTP whereas all other tumors were completely negative. In line with the findings of Swarts *et al*., OTP was neither expressed in normal tissues nor in other organs examined.[17] Neuroendocrine cells of the normal bronchus and bronchiole, identified with synaptophysin, were negative for OTP as well. Similar results were observed by Hanley *et al*., who evaluated immunohistochemical OTP expression in fine-needle aspiration (FNA) samples derived from extrapulmonary and pulmonary sites (Table 5.1). OTP was positive in 17% (n=10/59) of the cases, and all positive samples were NETs from either the lung or a metastasis from a primary lung tumor (Table 5.2).[18] None of the NETs derived from extrapulmonary sites showed any positivity for OTP. Among the 15 pulmonary carcinoids, 100% (n=9/9) of the TCs were positive compared to only 17% (n=1/6) of the ACs, indicating that OTP is preferably expressed within TCs (Table 5.2).[18] Nevertheless, Viswanathan *et al*., evaluated OTP expression in both pulmonary non-neuroendocrine and neuroendocrine tumors (Table 5.1).[21] According to the results, neither non-neuroendocrine tumors nor high grade neuroendocrine carcinomas stained positive for OTP. However, 82% (n=9/11) of TCs and 83% (n=10/12) of ACs showed positivity for OTP confirming the specificity of OTP for PCs (Table 5.2). Yet, in contrast to other studies, both TCs and ACs showed equal positivity towards OTP on fine

needle aspiration (FNA) specimen.[17,18,21] However, it should be noted that they did observe a significant difference in the degree of OTP staining in surgical resection material between TC and AC. 73% of TCs showed >40% OTP staining, whereas only 30% of ACs displayed >40% OTP staining, indicating that the staining used in this study was suboptimal for small biopsies.

Another recent study of Yoxtheimer *et al*., examined OTP IHC expression on 50 FNA specimens, including 30 primary pulmonary NENs (eight TCs, six ACs, five LCNEC, and 11 SCLC) and 20 primary pancreatic NETs (Table 5.1).[20] Results showed that 50% (n=4/8) of the pulmonary TCs expressed OTP, while merely 17% (n=1/6) of ACs and 20% (n=1/5) of LCNEC expressed OTP (Table 5.2). Moreover, neither SCLC nor any pancreatic NET expressed OTP.[20] Taken together these studies define OTP as a highly specific marker for pulmonary carcinoid disease. Since OTP turns out to be a highly specific marker, an increasing number of studies are starting to evaluate the diagnostic utility of OTP in tumors with NE differentiation. Recently, Roy *et al*., assessed OTP expression in tissue microarrays of 32 FFPE malignant tumors with neuroendocrine differentiation from the gynaecologic organs (n=16), breast (n=8), and prostate gland (n=6).[23] Nuclear expression of OTP was interpretable in 26 cases and detected in only 15% (n=4/26) including two prostate adenocarcinoma and two NE carcinomas of the ovary. OTP expression was absent in the remainder of the gynaecologic malignancies and NE mammary carcinomas. These data imply that though OTP is a very specific marker for NE lung carcinoids, it is not a sensitive broad-spectrum NE marker.

OTP in pulmonary neuroendocrine cell hyperplasia (NECH)

Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) was first described by Aguayo *et al*., as an unusual clinical entity that may cause airway fibrosis.[24] DIPNECH is confined to the respiratory epithelium layer without penetration of the basement membrane and is not related to any known predisposing condition.[25] Nowadays, it is known that pulmonary neuroendocrine cell hyperplasia (PNECH) can emerge, not only as a reaction to inflammation, but also in the context of carcinoid or adenocarcinoma development (Figure 5.1D).[26] In the latest WHO classification of lung tumors, DIPNECH was described as a generalized proliferation of pulmonary neuroendocrine cells that may be restricted to the mucosa of airways, may invade locally to form tumorlets, or might develop into carcinoid tumors.[4] Interestingly, we found that OTP is highly expressed in these speculated lung carcinoid precursors.[16] In a cohort study containing seven DIPNECH cases, Nonaka *et al*., showed that OTP was not expressed in normal tissues and organs whilst OTP was convincingly expressed in all DIPNECH lung resections (Table 5.2).

Afterwards, the same research group added an additional nine DIPNECH cases to their former research cohort which showed an ubiquitous OTP staining as well (Table 5.2).[19] A recent review illustrated the progression of disease in carcinoids and proposed that carcinoid tumors may arise from neuroendocrine cells and related neuroendocrine cell hyperplasia.[27] In addition, it was suggested that the genes OTP/CD44 might play an important role in carcinoid development.

OTP structure

OTP is a gene which encodes a member of the homeodomain (HD) family. A homeodomain is a 180-nucleotide DNA sequence that encodes a helix turn helix DNA binding domain, which was discovered in Drosophila flies during the mid-1980s by McGinnes *et al*..[28] Genes possessing a HD are transcriptional regulators which play key roles in the specification of cell fates. In an effort to identify human HD genes, Lin *et al*., successfully cloned the human homologue of the murine gene Orthopedia (*Otp*), which demonstrated 99% homology to mouse *Otp.*[29] The human gene is located at chromosome 5q14.1 containing three exons and two introns, two splice variants and a high GC-content (Figure 5.2). *OTP* encodes a protein composed of 325 amino acids and contains two protein domains namely a homeobox domain and an OAR-domain, the function of the latter is unknown (Figure 5.2).

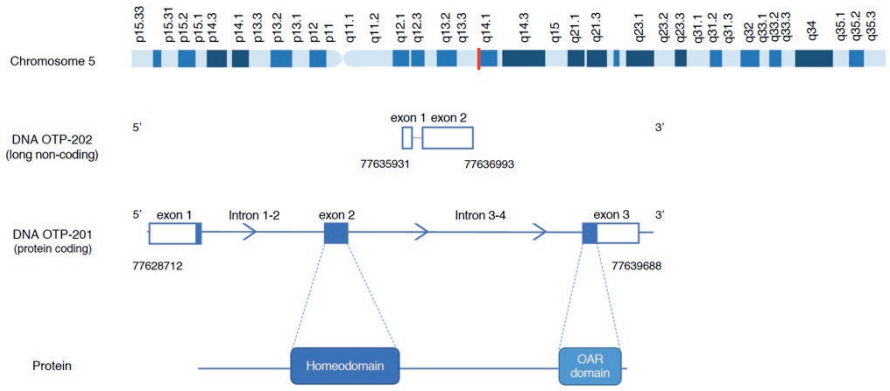

Figure 5.2 Schematic representation of the *OTP* gene. The chromosome row represents the genomic location of *OTP* (Red, 5q14.1), the DNA rows represent the gene composition with the genomic coordinates of both the protein coding transcript and the processed transcript, and the protein row represents the translated protein domains within the gene.

OTP function

OTP in the Hypothalamus

Although the specific function of OTP remains largely unknown, OTP has been frequently reported to be a key player in the development of the hypothalamic neuroendocrine system of vertebrates such as zebrafish, mice, and humans.[30-32] The hypothalamic neuroendocrine system is a crucial region in the brain which regulates homeostasis by mediating endocrine, autonomic, and behavioural functions. It comprises several nuclei containing distinct neuronal populations producing neuropeptides and neurotransmitters which regulate fundamental body functions.[33] In mice, Otp expression is well conserved in hypothalamic domains and involved in the differentiation of several neurohormone secreting nuclei including the anterior periventricular, paraventricular, supraoptic, arcuate nuclei.[31,34,35] Neurogenesis of the endocrine hypothalamus is characterized by a series of crucial developmental milestones such as the initial commitment to the neuronal fate, neuroblast proliferation, migration of postmitotic neurons to the neuroendocrine nuclei, and terminal differentiation including neuropeptide expression and axonal outgrowth.[32] Analysis of *Otp* knockout (*Otp*-/-) mice revealed that Otp is able to affect all developmental milestones except for the initial commitment to the neuronal fate, indicating an essential role for Otp in proper murine neuroendocrine hypothalamic development.[32,34] Nevertheless, the development of neuroendocrine cell lineages in the hypothalamus requires, besides OTP, additional transcription factors. Acampora *et al*., showed that OTP is coexpressed with single-minded-homology 1 (SIM1) in the same cells at the same time.[32] In addition, they showed that Otp acts upstream of Brn-2, a developmental neural cell-specific POU domain transcription factor (POU3F2). By using single-minded-homology 1 (*Sim1*) mutant mice, Acampora *et al*., showed that both Otp and Sim1 are required for POU3F2 expression (Figure 5.3). To summarize, OTP is a highly conserved transcription factor which regulates the fate, migration, and terminal differentiation of hypothalamic neurons.

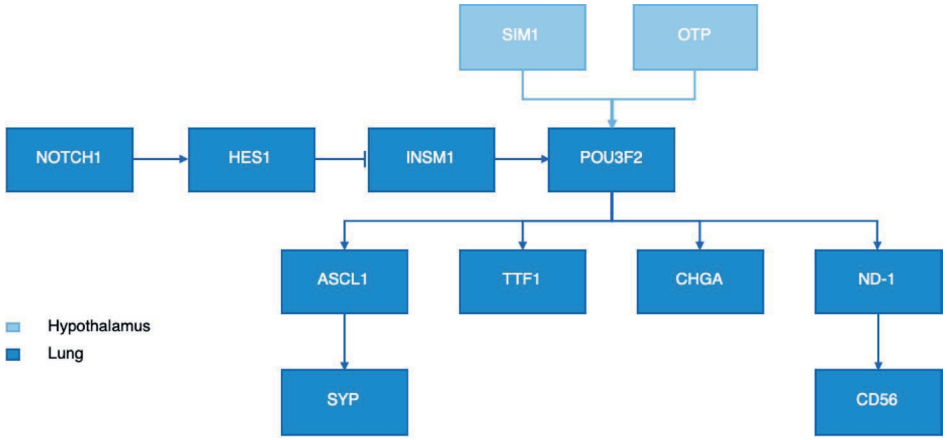

Figure 5.3 Proposed schematic overview of the molecular network through which OTP might act along with other neuroendocrine (NE) related factors in the hypothalamus and the lungs.

OTP in lung neuroendocrine tumors

A recent study of Nonaka *et al*., proposed a link between OTP and thyroid transcription factor 1 (TTF1).[17] The expression of TTF1, a member of the homeodomain transcription factor family, is one of the most essential IHC stains in the diagnostic histopathology of lung and thyroid tumors.[36] Immunohistochemical analysis on 162 pulmonary carcinoids revealed that all TTF1 positive tumors were also positive for OTP.[17] On the other hand, none of the OTP negative tumors stained positive for TTF1. Additionally, neither OTP nor TTF1 was expressed in normal NE cells.[15,19] Taken together, we propose that OTP might be upstream of TTF1 in the transcription factor hierarchy. Nevertheless, Papaxoinis *et al*., and Hanley *et al*., described cases positive for TTF1 whilst negative for OTP suggesting the presence of intermediate factors in PCs.[17,19] One of these intermediate factors might be the downstream target of OTP, POU Class 3 Homeobox 2 (POU3F2), since a recent study proposed a crucial role for POU3F2 in the expression of lineage-specific transcription factors such as achaete-scute homolog-like 1 (ASCL1) and NeuroD1 (ND1) and NE marker molecules like neural cell adhesion molecule 1 (NCAM1), synaptophysin (SYP), and chromogranin A (CHGA) in SCLC (Figure 5.3).[37] In addition, Sakaeda *et al*., reported that POU3F2 is directly involved in TTF1 expression in SCLC.[38]

A possible explanation for the OTP+/TTF1− cases might be the involvement of the NOTCH1-HES1 signalling pathway which is reported as an inhibitor of ASCL1, POU3F2, and NE molecules (CHGA, CD56, SYP).[39] NOTCH receptor 1 (NOTCH1) is known to activate hairy and enhancer of split-1 (HES1), which inactivates insulinoma-associated

protein 1 (INSM1), ASCL1, and POU3F2 (Figure 5.3).[40] Nevertheless, recent studies described that Notch1 signalling is minimal or even absent in pulmonary TC and AC and gut carcinoids.[40-44] As a result, the transcription factor HES1 will be inactive and INSM1 will not be inhibited leading to the activation of the transcription factors ASCL1 and POU3F2 which promote the expression of NE molecules such as TTF1 (Figure 5.3). Although the pathway through which OTP acts remains largely unknown, here we speculate a schematic overview of downstream targets of OTP and the possible involvement of the NOTCH1 pathway.

OTP DNA analysis

Several studies extensively profiled PCs to obtain more insights into the molecular characteristics of these rare entities.[27,45-49] Recently, Alcala *et al*., performed multiomics (genome, exome, transcriptome and methylome) integrative analyses on 116 PCs.[50] Carcinoids were classified into different clusters based on multiomics cluster analysis (MOFA) of which cluster carcinoid A was enriched for TCs (75%) whilst cluster carcinoid B was enriched for ACs (54%).[50] Cluster B showed the worst survival and was characterized by the universal downregulation of *OTP* (90% with fragments per kilo million (FPKM) <1). In addition, they showed that these expression levels of *OTP* were strongly correlated with survival which is in line with previous studies.[14,15] Despite the high expression difference between favourable versus poor survival, the regulatory mechanism of *OTP* remains to be further investigated.

Nowadays, patterns of somatic mutations caused by different mutational processes in cancer genomes have been identified as the result of advances in genome sequencing and the development of computational tools. Multiple studies have performed mutational analysis on pulmonary carcinoid tumors.[45-49] Though carcinoids are characterized by a low number of nonsynonymous mutations per million base pairs, most frequently mutated genes are implicated in chromatin remodelling. In addition, histone modifiers and members of switch/sucrose non-fermentable (SWI-SNF) complexes are mutated in approximately 40% and 22%, with multiple endocrine neoplasia type 1 (MEN1) most frequently affected.[45,47,51] However, until now no mutations have been identified in *OTP*, suggesting the presence of additional mechanisms leading to substantial expression differences.

Besides genetic changes in DNA and chromosomes, it has become evident that oncogenomic processes can be profoundly influenced by epigenetic mechanisms. DNA methylation is a major epigenetic factor involved in the regulation of gene expression and refers to the addition of a methyl group to the fifth position of a cytosine.[52] Nowadays, numerous human diseases have been linked to aberrant DNA methylation of which

hypermethylation of CpG islands in the promotor region has been most extensively studied in cancer. Currently, various genes with aberrant promotor hypermethylation have been identified in all forms of cancer. While several studies have explored promotor methylation in pulmonary carcinoids, to date, only Alcala *et al*., investigated the whole methylome of 56 carcinoids (33 TCs and 23 ACs) using 850K arrays.[50] Gene expression and corresponding promotor methylation data were correlated to identify genes which expression could be explained by their methylation pattern. While one of the top correlations was found for *HNF1A* and *HNF4A* homeobox genes, no correlation was found for *OTP.*[50]

In summary, different *OTP* mRNA and protein expression levels are found to be related to prognosis. However, the exact mechanism of OTP (in) activation has not been identified yet.

Conclusions

We provide a comprehensive overview of available literature demonstrating OTP as a promising, highly sensitive, and specific marker for pulmonary carcinoid tumors with a favourable prognosis. Nuclear OTP in combination with CD44 protein expression may be used as a predictive marker to exclude patients having a very low risk for distant recurrence of carcinoid disease from long term follow-up. Nevertheless, additional cohort studies focusing on disease free survival are necessary to implement OTP in routine diagnostics. Besides, the underlying mechanism regulating OTP in neuroendocrine pulmonary (tumor) cells remains to be elucidated. Hence, future studies should be focused on unravelling the interplay between regulation of OTP expression, and its biological role in the downstream aggressive behaviour of these pulmonary neuroendocrine lesions.

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CHAPTER 6

OTP, CD44, and Ki-67: A prognostic marker panel for relapse free survival in patients with surgically resected pulmonary carcinoid

L. M. Sonen, J. L. Derks, M. A. den Bakker, L. M. Hillen, R. J. van Suylen, J. H. von der Thüsen, L. M. V. Lap, B. J. C. A. Marijnissen, R. A. Damhuis, K. M. Smits, E. C. van den Broek, W. A. Buikhuisen, PALGA group, A-M. C. Dingemans, E. J. M. Speel

Submitted

CHAPTER 7

Differential Orthopedia Homeobox (OTP) expression in pulmonary carcinoids is associated with changes in DNA methylation

L. Moonen*, L. Mangiante*, D. J. G. Leunissen, L. M. V. Lap, A. Gabriel, L. M. Hillen, G. M. Roemen, A. Koch, M. van Engeland, A-M. C. Dingemans, M. Foll, N. Alcala, L. Fernandez-Cuesta**, J. L. Derks**, E. J. M. Speel**
*Equally contributing first authors, **Equally contributing last authors
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Abstract

Limited number of tumor types have been examined for Orthopedia Homeobox (OTP) expression. In pulmonary carcinoids, loss of expression is a strong indicator of poor prognosis. Here, we investigated *OTP* expression in 37 different tumor types, and the association between *OTP* expression and DNA methylation levels in lung neuroendocrine neoplasms. We analysed publicly available multi-omics data (whole-exome-, whole-genome-, RNA sequencing, Epic-850K-methylation array) of 58 typical-, 27 atypical carcinoids, 69 large cell neuroendocrine carcinoma and 51 small cell lung cancer patients and TCGA (The Cancer Genome Atlas) data of 33 tumor types. 850K-methylation analysis was cross validated using targeted pyrosequencing on 35 carcinoids. We report bimodality of *OTP* expression in carcinoids (OTP^{high} versus OTP^{low} group, likelihood-ratio test $p=1.5x10^{-2}$), with the OTP^{high} group specific to pulmonary carcinoids while absent from all other cohorts analysed. Significantly different DNA methylation levels were observed between OTP^{high} and OTP^{low} carcinoids in 12/34 OTP infinium probes (fdr<0.05 & β-value effect size>0.2). OTP^{low} carcinoids harbour high DNA methylation levels as compared to OTP^{high} carcinoids. OTP^{low} carcinoids showed a significantly worse overall survival (logrank test $p=0.0052$). Gene set enrichment analysis for somatically mutated genes associated with hallmarks of cancer showed robust enrichment of three hallmarks in the OTP^{low} group, i.e., sustaining proliferative signalling, evading growth suppressor, and genome instability and mutation. Together our data suggest that high *OTP* expression is a unique feature of pulmonary carcinoids with a favourable prognosis and that in poor prognostic patients, *OTP* expression is lost, most likely due to changes in DNA methylation levels.

Introduction

Lung neuroendocrine neoplasms (LNENs) comprise a heterogeneous group of malignancies, mainly arising from pulmonary neuroendocrine cells, which account for approximately 20 percent of all primary lung cancers. According to the World Health Organization (WHO) 2021 classification, LNENs are subdivided into four different entities namely, typical carcinoids (TC, 1.8%), atypical carcinoids (AC, 0.2%), large-cell neuroendocrine carcinomas (LCNEC, 3%), and small-cell lung carcinomas (SCLC, 15%).[1,2] Even though pulmonary carcinoids only encompass 1-2% of all invasive lung malignancies, their occurrence has increased significantly over the past decades.[3,4]

LNENs share morphological and immunohistochemical features but they show a broad clinical pathological spectrum with a different biological behaviour. In contrast to the high-grade LNENs (e.g., LCNEC and SCLC), carcinoids are characterized by a low mitotic frequency ($\leq$10 mitosis/mm^2), none or punctuated necrosis, and a relatively favourable prognosis. Although carcinoids are considered as low- or intermediate grade tumors, they may show distant disease relapse (TC: 1-6% and AC: 14-29%) in patients who initially underwent curative surgical resection.[5,6]

Known predictive factors for recurrence of disease after surgery for carcinoids are histopathological type (AC), tumor size, and lymphatic involvement. Unfortunately, none of these factors enable the clinician to reliably predict patients at risk for recurrence up-front. Therefore, extensive follow-up is required for all patients with a carcinoid up to 15-year after surgical resection resulting in frequent oversurveillance.[1,7,8] A possible alternative is to subdivide carcinoids into prognostically relevant categories (at risk for recurrence versus no risk) using tumor specific molecular features. Previously, we identified Orthopedia Homeobox (OTP) as a prominent molecular marker to accurately identify patients at risk for disease recurrence and showed that *OTP* transcription levels and nuclear protein expression levels were strongly correlated (p=5.9e^{11}, n=60).[9] In addition, our research group and others have shown that OTP protein expression is absent in normal neuroendocrine cells of the lung, whereas OTP is highly expressed in carcinoid precursor lesions (i.e., neuroendocrine cell hyperplasia and tumor lets), as well as in most pulmonary carcinoids.[9-14] Other NENs (i.e. gastrointestinal/pancreatic) turned out to be OTP negative [10]. Intriguingly, pulmonary carcinoids without expression of OTP at the time of diagnosis develop more frequently metastases during follow-up.[11] These data suggest that OTP is a prognostic marker in pulmonary carcinoids and may play a role in carcinoid tumorigenesis. Although OTP has been described as a key player in the development of the hypothalamic neuroendocrine system of vertebrates, its specific role in tumorigenesis and carcinoid disease recurrence remains to be elucidated.

Here, we performed comparative analysis on *OTP* expression throughout 37 different tumor types, using publicly available The Cancer Genome Atlas (TCGA) data (*n*=33/37 tumor types) combined with publicly available LNEN data (*n*=4/37 tumor types), to identify groups with similar expression profiles. Subsequently, we used multi-omics publicly available data on LNENs to study the molecular differences between OTP^{high} versus OTP^{low} LNENs, with special focus on carcinoids.[15] In addition, we correlated transcriptomic and methylomic data to shed light on the regulatory mechanisms of OTP expression.

Materials and methods

Public LNEN and TCGA data

LNEN whole-exome sequencing (WES), transcriptome sequencing (RNAseq), Epic 850K Illumina arrays methylation data, mutational data and corresponding clinical and histopathological annotations were available from the lungNENomics project within the Rare Cancers Genomics initiative http://rarecancersgenomics.com/lungnenomics/. Detailed information on data generation and quality controls can be found in Gabriel *et al*. GigaScience 2020.[16] This dataset includes 88 carcinoids of which 58 TC, 27 AC and 3 unclassified carcinoids, 69 LCNEC, and 51 SCLC.[15,17-19] All samples were collected from surgically resected tumors.

Transcriptomic data (In units of Fragment Per Kilobase of transcript per Million mapped read, FPKM) and corresponding clinical data of 33 different tumor types were gathered from the publicly available The Cancer Genome Atlas (TCGA) platform. Data was downloaded using the Bioconductor R package *TCGAbiolinks* (version 2.9.5). Duplicated samples were removed as well as one lung squamous cell carcinoma sample (TCGA-37-4129) because this sample was previously reported as misclassified.[20] TCGA *OTP* expression data was merged with LNEN *OTP* expression data to gather an overview of *OTP* expression patterns throughout 37 different tumor types.

Data processing of expression data

Data processing was performed as described previously by Alcala, et al.[15], providing quantification of expression at the gene level in two formats. (i) FPKM, one of the most popular formats for expression quantification, that facilitates comparisons across cohorts by mitigating technical batch effects through normalization based on gene length and sequencing library size; this is the format we used for comparing with TCGA cohorts, and for interpreting absolute expression levels. (ii) Normalized read counts, obtained through

the variance stabilization procedure described in Alcala et al. using the *DESeq2* R package[15], that facilitates downstream statistical analyses of the expression data within a cohort by reducing the relationship between mean and variance in expression level; this is the format we used for the statistical analysis of the expression (see determining an expression cut-off point procedure, and gene set enrichment analysis below).

Clinical data

Clinical cohort data were retrieved from Alcala et al..[15] Data included, amongst others, age (in years), sex (male or female), smoking status (never smoker, former smoker, passive smoker, and current smoker), Union for International Cancer Control/American Joint Committee on Cancer stage (Stage I to IV), and survival (calculated in months from surgery to last day of follow-up or death).

Gene-set enrichment analysis of somatic mutations

Gene-set enrichment analysis of somatic mutations (GSEA) was performed as described previously.[15] In short, GSEA for somatic mutations was evaluated independently for each set of genes linked to a hallmark of cancer, taking into account genes with multiple mutations, using the Fisher's exact test (Fisher.test R function, *stats* package version 4.0.4).[21] P-values for both the OTP^{high} and OTP^{low} group were adjusted for multiple testing. Altered hallmarks, including the mutated genes are presented in Supplementary Table S7.1.

In addition, we performed robustness analyses to assess the validity of the results, especially to outlier samples/genes that would have a high influence on the statistical results, i.e., that would alone drive the significance of a particular hallmark. First, we assessed the influence of each individual sample using a jackknife procedure (i.e., for each sample, we performed the GSEA test after removing this sample). Second, we assessed the influence of each gene using a jackknife procedure (i.e., for each gene, we performed the GSE test without this gene). A threshold adjusted *p*-value of 0.05 was fixed to select enriched pathway.

Methylome analyses

DNA methylation levels at each OTP Infinium probe (publicly available 850K array data) were analysed for 51 LNEN samples (*n*=19 TC, *n*=15 AC, *n*=17 LCNEC) of the total cohort, combined with 528 publicly available TCGA normal lung adenocarcinoma (*n*=274) and squamous cell carcinoma (*n*=254) tissues (both 450K array data). Differential DNA methylation was tested using the Wilcoxon rank sum test on β-values, to allow

comparisons between the non-normally distributed distributions of β-values. False discovery rate (FDR) was controlled using the Benjamini-Hochberg procedure to correct for multiple hypothesis testing. In addition, an overview of the DNA methylation data at all OTP probes together with the genomic location of these probes was presented. The exon and transcript annotations were retrieved from Ensembl using the BioMart tool (Ensemble Genes 75, Homo sapiens genes GRCh38.p12), CpG island locations were downloaded from the UCSC genome browser[22], and the EPIC probe locations were retrieved from Zhou *et al*. (2017) using the human genome version GRCh38.p12.

Validation of the methylome analyses using pyrosequencing

To cross-validate the methylome analyses findings, we have performed pyrosequencing on *n*=35/88 fresh frozen carcinoid samples of the sequencing cohort. DNA was isolated using the Gentra Puregene tissue kit 4g (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA samples were quantified by the fluorometric method (Quanti-iT PicoGreen dsDNA Assay, Life Technologies, CA, USA) and integrity was checked by gel electrophoresis using a 1.3% agarose gel. Next, sodium bisulfite-modification, which converts unmethylated cytosine residues to uracil residues, was performed on 100 ng genomic DNA using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Bisulfite modified DNA was amplified using methylation specific primers, which were designed using the Pyromark Assay design SW 2.0 (Qiagen, Hilden Germany) (Table 7.1). PCR amplification was performed using the PyroMark PCR kit (Qiagen, Hilden Germany), according to the manufacturer's protocol, on a T100^{TM} thermal cycler (Bio-Rad). Afterwards, PCR products were loaded on a 2% agarose gel and visualized using Proxima AQ-4 imaging software (Isogen, De Meern, The Netherlands). Next, the methylation status of cg26576712 (genomic location 77640156-77640158) and cg02493167 (genomic location 77639893-77639895), both located in the promoter region of *OTP*, were determined by pyrosequencing using PyroMark Q24 Advanced CpG reagents and a PyroMark Q24 Instrument upgraded with the PyroMark Q24 Advanced software (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After pyrosequencing, the methylation percentage of each site was determined by analysing the pyrograms using the PyroMark Q24 Advanced software (Qiagen, Hilden, Germany). Every PCR and pyrorun included a bisulfite converted and methylated DNA obtained from a RKO cell line, EpiTect unmethylated and bisulfite-converted controls (Qiagen, Hilden, Germany), and a H_2O control. In addition, an internal cytosine control was incorporated in the sequence to be analysed. Pyrosequencing on normal lung samples (*n*=3) was performed to determine the cut-off methylation level on cg26576712 and cg02493167 (mean positivity threshold 32.2%).

Table 7.1 Overview of the primer combinations for both CpG sites within the promoter region of OTP and their specifications.

Location	Primer	Sequence	Nt	Tm °C	% CG	Product length (bp)
OTP cg02493167	Forward	GGGAGTAGTAAATATTAGTTTTTATTGTGA	30	58.8	26.7	160
	Reverse	ATTCTATACCATTTCTAATCTACTCCTAAA	30	57.3	26.7	
	Pyrosequencing	ATGTTTTGTTATAAATATAATTG	23	39.2	13.0	
OTP cg26576712	Forward	GTTTTTAGTTAGTATTTTTAATGTTTTGTTTAAGT	35	57.2	17.1	116
	Reverse	CCTTCCACAAAAAAATAACCCAATAA	26	58.4	30.8	
	Pyrosequencing	ATGTTTTGTTTAAGTTAATTGG	22	44.6	22.7	

Abbreviations: bp, base pairs; CG, cytosine-guanine content; Nt, nucleotides; Tm, melting temperature.

Immunohistochemistry

Immunohistochemistry (IHC) on formalin fixed paraffin embedded (FFPE) tissue sections was performed using the rabbit anti-OTP primary polyclonal antibody (HPA039365; Atlas Antibodies). The primary antibody was diluted 1:3000 and incubated overnight at 4 degrees Celsius. Antibodies were detected by Bright Vision Poly-HRP-anti-mouse/rabbit/rat immunoglobulin G (IgG; Immunologic) followed by peroxidase-DAB (3,3'-diaminobenzidine) visualization. Expression of OTP IHC was scored as described previously.[9]

Statistical analysis

Statistical analyses were conducted using SPSS for Mac version 25 (SPSS Inc., Chicago, IL, USA) and RStudio for Mac version 3.6.1. The chi-squared test and Fisher's exact test were used to compare categorical data. A one-sample t-test was performed to test the significance between measures in the bland-altman plots. Survival analysis for LNENs was performed using Cox's proportional hazard model (R package *survival* version 3.2-11), with statistical differences between groups performed using the Wald test and global fit of the model assessed using the logrank test. Two-sided *p*-values <0.05 were considered significant. Bimodality in gene expression data was tested using the function normalmixEM from the R package *mixtools*. A likelihood ratio test was performed, using the chi-squared test to choose between a null model (unimodal Gaussian distribution) and the bimodal model, rejecting the null when *p*-value<0.05. The optimal cut-off was then defined as the lowest density point of the two Gaussian mixture distributions (in our case, 8.7).

Independent dataset of pulmonary carcinoids

To confirm our findings, we used the publicly available gene expression, mutation, and Illumina 450K methylation array data from an independent pulmonary carcinoid cohort

(Laddha et al., 2019). The cohort comprised of 30 primary lung carcinoids including 17 TC and 13 AC. Methylome data were available in 17 patients (*n*=10 TC and *n*=7 AC). The data were downloaded from supplementary data files (https://www.omicsdi.org/dataset/geo/GSE118133) reported in ref..[23] Data processing and subsequent analysis were performed as previously described above.

Results

Patterns of OTP expression in pulmonary carcinoids

To gain insights into the expression patterns of *OTP* throughout 33 different TCGA tumor types and four LNEN subtypes, we performed comparative data analyses. Results showed that *OTP* was expressed in pulmonary carcinoids with higher levels in TC (median 126.4, Interquartile range (IQR) 72.9–193.4 FPKM) than in AC (median 0.16, IQR 0.06-57.7 FPKM). However, both TC and AC groups include samples with lower and higher *OTP* expression, respectively (Figure 7.1A). All other tumor types, including pulmonary LCNEC (median 0.09, IQR 0.02–0.17 FPKM) and SCLC (median 0.19, IQR 0.08–0.55 FPKM), showed very low to no *OTP* expression. Both the glioblastoma (GBM) (median 0.64, IQR 0.19–1.7) and low-grade glioma (LGG) (median 0.007, IQR 0.001–0.04) cohort show an overall low median expression of *OTP*, some samples within these groups display higher *OTP* expression levels (Figure 7.1A). Nevertheless, these data suggest that *OTP* is a highly specific marker for pulmonary carcinoids as compared to all other TCGA tumor types.

Considering that high- and low-*OTP* expression samples were detected in both TC and AC, we assessed the *OTP* gene expression of 88 sequenced carcinoids to determine the optimal cut-off point for OTP^{high}- and OTP^{low} -classification. A mixture model of two Gaussian distributions was fitted to the distribution of *OTP* expression (normalized read counts) in LNENs (Figure 7.1B). Data showed a clear bimodal distribution of *OTP* in carcinoids ($p=1.5x10^{-2}$) suggesting that within the carcinoid cohort, two distinct groups exist, which can be separated based on *OTP* expression levels (Figure 7.1B, upper panel). Next, we confirmed the absence of *OTP* bi-modality in both the LCNEC- and SCLC sample cohort (Figure 7.1B; middle and bottom panels, respectively). By applying this cut-off point (lowest density point of the two Gaussian mixture distributions 8.7, in units of normalized read counts) to the high grade LNENs, only one LCNEC sample and one SCLC sample were considered OTP^{high}, whereas all other high-grade LNENs were classified as OTP^{low}. In fact, Alcala et al. recently showed that this LCNEC sample (S00602) clustered with carcinoid tumors according to Multi-Omics Factor Analysis (MOFA) [15].

Molecular and clinical characteristics of the OTP^{high} and OTP^{low} carcinoids

Baseline clinical characteristics are presented in Table 7.2. The OTP^{high} cohort was enriched for females (69%), while the OTP^{low} cohort contained relatively more males (79%, Fisher's exact test *p*-value=7.9x10^{-5}). Both age and smoking status were comparable within the two groups (Fisher's exact test both *p*-value=0.16). In addition, patients in the OTP^{high} group were more frequently diagnosed as TC, whereas the OTP^{low} group was enriched for AC histopathology (Fisher's exact test *p*-value=0.00013). Compared to OTP^{low}, patients in the OTP^{high} group were more frequently diagnosed in lower Tumor Node Metastasis (TNM) stages (Fisher's exact test of stage I-II vs. stages III-IV *p*-value=0.01).

Kaplan-Meier Survival analysis was performed for the pulmonary carcinoids clustered in OTP^{high} and OTP^{low}. Results showed that carcinoids with low *OTP* expression harbour an unfavourable survival as compared to carcinoids with high *OTP* expression (logrank test *p*-value=0.0052, Figure 7.1C).

In order to investigate whether the survival difference between OTP^{high} and OTP^{low} carcinoids might be associated with somatic gene mutations, we performed a GSEA for mutated genes related to hallmarks of cancer.[21] The analysis showed that mutated genes in carcinoids clustered in the OTP^{low} group were strongly enriched in three hallmarks, i.e., sustaining proliferative signalling (*p*=1.05x10^{-3}), evading growth suppressor (*p*=1.11x10^{-8}) and genome instability and mutation (*p*=6.53x10^{-5}, Supplementary Figure S7.1, Supplementary Table S7.1). Jackknife analyses of samples showed that OTP^{low} enrichments for somatic alterations were not influenced by a single sample (Supplementary Table S7.1).

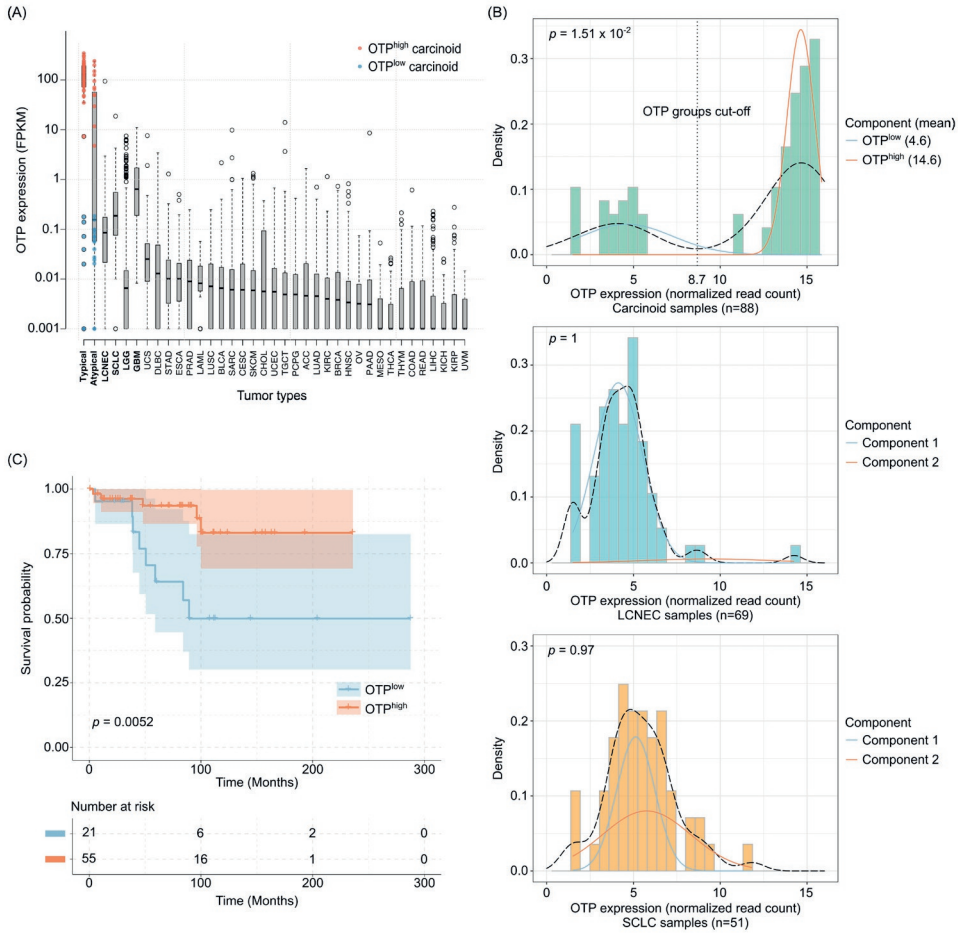

Figure 7.1 (**A**) RNA gene expression of OTP in 37 different tumor types highlighted using boxplots in fragment per kilobase million (FPKM). Center lines represent the median and box bounds represent the interquartile range (IQR). (See Table S7.4 for the abbreviation list of the tumor types.) (**B**) Histograms presenting the OTP expression pattern, in units of normalized read counts, in carcinoids (upper panel), LCNEC (middle panel) and SCLC (lower panel). The striped curve represents the distribution fit of the two Gaussian mixture distributions (component 1, in blue and component 2, in orange). The OTP cut-off is determined as the lowest density point of the two Gaussian mixture distributions (upper panel, x = 8.7). (**C**) Kaplan-Meier curves of overall survival probability for the OTP^{high} and OTP^{low} group in pulmonary carcinoid patients.

In addition, jackknife analyses of mutated genes underlined that *MEN1* is necessary for the enrichment of genome instability and mutation and sustaining proliferative signalling hallmarks in the OTP^{low} group (Supplementary Table S7.1). In the OTP^{high} cluster three hallmark enrichments nearly reached significance including activating invasion and metastasis (p=0.03), evading growth suppressor (p=0.02), and sustaining proliferative

signalling (p=0.04, Supplementary Table S7.1). However, it must be considered that jackknife analyses for both samples and mutated genes in the OTP^{high} group revealed poor stability of enrichments, indicating that the enrichments are driven by specific samples and/or mutated genes. Interestingly, *MEN1* mutations were found in six out of the 14 carcinoids clustered in the OTP^{low} group while only one case with a *MEN1* mutation was found in the OTP^{high} group of 40 patients. This is in agreement with several studies proving that *MEN1* mutations are associated with poor prognosis.[17,24,25] These data suggest that the clear and robust enrichment of hallmarks of cancers in the OTP^{low} group may explain tumor aggressiveness and thereby the difference in survival.

Table 7.2 Patient characteristics of the OTP^{high} and OTP^{low} group.

Variable	Groups		*p*-value
	OTP^{high}	OTP^{low}	
Patients n	64	24	
Age years			
Mean +/- SD	51,6 ± 18,3	58,3 ± 12,8	
Median IQR	54 (16-80)	58 (29-80)	0.16
Gender			7.9×10^{-5}
Female	44 (68.75)	5 (20.8)	
Male	20 (31.25)	19 (79.2)	
Smoking status			0.16
Current	14 (21.9)	3 (12.5)	
Former	13 (20.3)	8 (33.3)	
Never	23 (35.9)	6 (25.0)	
Passive		1 (4.2)	
Histopathological classification			$1{,}30 \times 10^{-4}$
Typical	50 (78.1)	8 (33.3)	
Atypical	12 (18.8)	15 (62.5)	
Unclassified	2 (3.1)	1 (4.2)	
TNM Stage			0.01
I – II	60 (94)	18 (75.0)	
III – IV	3 (5)	6 (25.0)	
Unknown	1 (1)		
Survival Censor			0.03
Alive	50 (78.1)	14 (58.3)	
Death	6 (9.4)	8 (33.3)	
Unknown	8 (12.5)	2 (8.3)	
Median survival in months	79,3	59	

Abbreviations: IQR, interquartile range; SD, standard deviation; TNM, tumor Node Metastasis.

Mechanisms of OTP inactivation

Genomic OTP inactivation

To examine which underlying regulatory mechanism may cause the expression differences between OTP^{high} and OTP^{low} carcinoids, we investigated publicly available gene mutation data.[15] Results to date have shown no gene-inactivating somatic

mutations, alterations by chimeric transcripts, or genomic rearrangements in the *OTP* gene (Supplementary data Alcala *et al.*[15]).[17]

To further investigate the regulatory mechanism underlying *OTP* expression, we evaluated a possible association between DNA methylation and *OTP* expression using using both transcriptome and methylome data (illumina Infinium 850K for tumor samples and 450k for normal samples). Transcriptomic and methylomic data were available in 51 samples including 24 OTP^{high}, 10 OTP^{low}, and 17 LCNEC samples. Results showed 12/34 OTP Infinium probes harbouring a significantly different methylation level (fdr<0.05 & delta>0.2) between OTP^{high} and OTP^{low} carcinoids, of which three probes were located in the promotor region (cg02493167, cg26576712, cg01763890) (Figure 7.2A, Supplementary Table S7.2). At these 12 loci, OTP^{high} carcinoids harbour a lower methylation level (based on β-values) as compared to OTP^{low} carcinoids. In addition, normal TCGA lung samples showed a baseline methylation level which was greater than OTP^{high} and lower than OTP^{low} carcinoids (Figure 7.2A, specified in green). This is interesting since to date, no OTP expression have been reported in normal lung tissues. Evaluation of the LCNEC samples showed a median RNA expression level of 0.09 FPKM and high methylation levels (Supplementary Figure S7.2). Of note, the methylation level of LCNEC samples was higher as compared to OTP^{low} carcinoids. Hence, we hypothesized that epigenetically hypermethylation of *OTP* could lead to gene-silencing and subsequent loss of protein expression.

To cross-validate the *OTP* methylation within the groups of pulmonary carcinoids, we performed pyrosequencing targeting two single promoter probes present in the Infinium 850K assay (cg02493167 and cg26576712). We have targeted these two CpGs since it has been frequently described that methylation within the promoter region of genes is associated with transcriptional silencing. Results revealed that 26% (*n*=9/35) of patients harboured an increased methylation status while 74% (*n*=26/35) showed no or low methylation percentages on cg02493167, using the methylation percentage of normal lung samples as the methylation threshold (average methylation percentage of 32.3%). Targeting cg26576712 showed an increased methylation status in 8/23 (35%) of the pulmonary carcinoids while 15/23 (65%) showed a low methylation percentage. 12 cases failed due to technical issues. Additional IHC analysis (*n*=14) confirmed loss of OTP expression in OTP-methylated carcinoids while expression was present in unmethylated carcinoids (Figure 7.2B). Bland altman plots showed no proportional difference between pyrosequencing and 850K arrays (Supplementary Figure S7.3; *p*=0.562 for cg26576712 and *p*=0.069 for cg02493167).
The identified association between DNA methylation and *OTP* expression raised the question whether one or multiple CpGs are associated with expression. For this purpose,

we have generated a heatmap of the β-values of all cg-sites for both carcinoid clusters (i.e., OTP^{high} and OTP^{low}) and LCNEC (Figure 7.2C). Complete-linkage clustering using the euclidean distance metric revealed a cluster with cgs that strongly correlates when applying a tree height cut-off of three (Figure 7.2C, cg21907107, cg16703762, cg02086801, cg26365545, cg17374364, specified in green). These cgs might possibly explain together the regulation of OTP expression. Noteworthy is cg17374364, which shows a β-value above 0.5 in all OTP^{low} samples (Figure 7.2C). While OTP^{high} samples show overall a low methylation level, data reveal that some samples (e.g., LNEN013, LNEN014, S01502, S01539) tend to show a higher methylation level (β-value >0.5). Somatic mutation analysis revealed that these samples harboured mutations in genes associated with Gene Ontology (GO) terms related to histone demethylase activity (HDM i.e., *KDM4A*, *KDM5C*, *FBXL19*) and histone methylation (HMT i.e., *DOT1L*) (Figure 7.2C). In addition, all cases tested with OTP IHC expression consistently showed that the OTP expression groups matched methylation groups (Supplementary Figure S7.4).

Independent confirmation of our findings

We verified our findings using published pulmonary carcinoid data from Laddha et al.[23], which included gene expression, mutation, and Illumina 450K methylation array data of 30 samples (17 TCs and 13 ACs). Bimodality testing confirmed our previous findings showing a clear bimodal distribution of *OTP* expression within the group of carcinoids (Supplementary Figure S7.5A, p=6.9x10^{-9}). Following the OTP cut-off (lowest density point of the two Gaussian mixture distributions 10.2, in units of normalized read counts), 7 patients were allocated to the OTP^{low} group and 23 patients to the OTP^{high} group. To investigate whether *OTP* expression is also associated with methylation in this cohort, we have generated a heatmap using the Illumina 450K methylation array data. Results showed that the highest methylation levels, in β-values, were observed in carcinoids clustered in the OTP^{low} group (Supplementary Figure S7.5B). Results showed that, in line with our findings described above, all OTP^{low} samples harbour a β-value above 0.5 on cg17374364, while OTP^{high} samples show overall a low methylation level (i.e., a β-value below 0.5). Furthermore, we observed a variable methylation level throughout all samples for cg05832593 (Supplementary Figure S7.5B, specified in red), also consistent with our data (Figure 7.2C, specified in red).

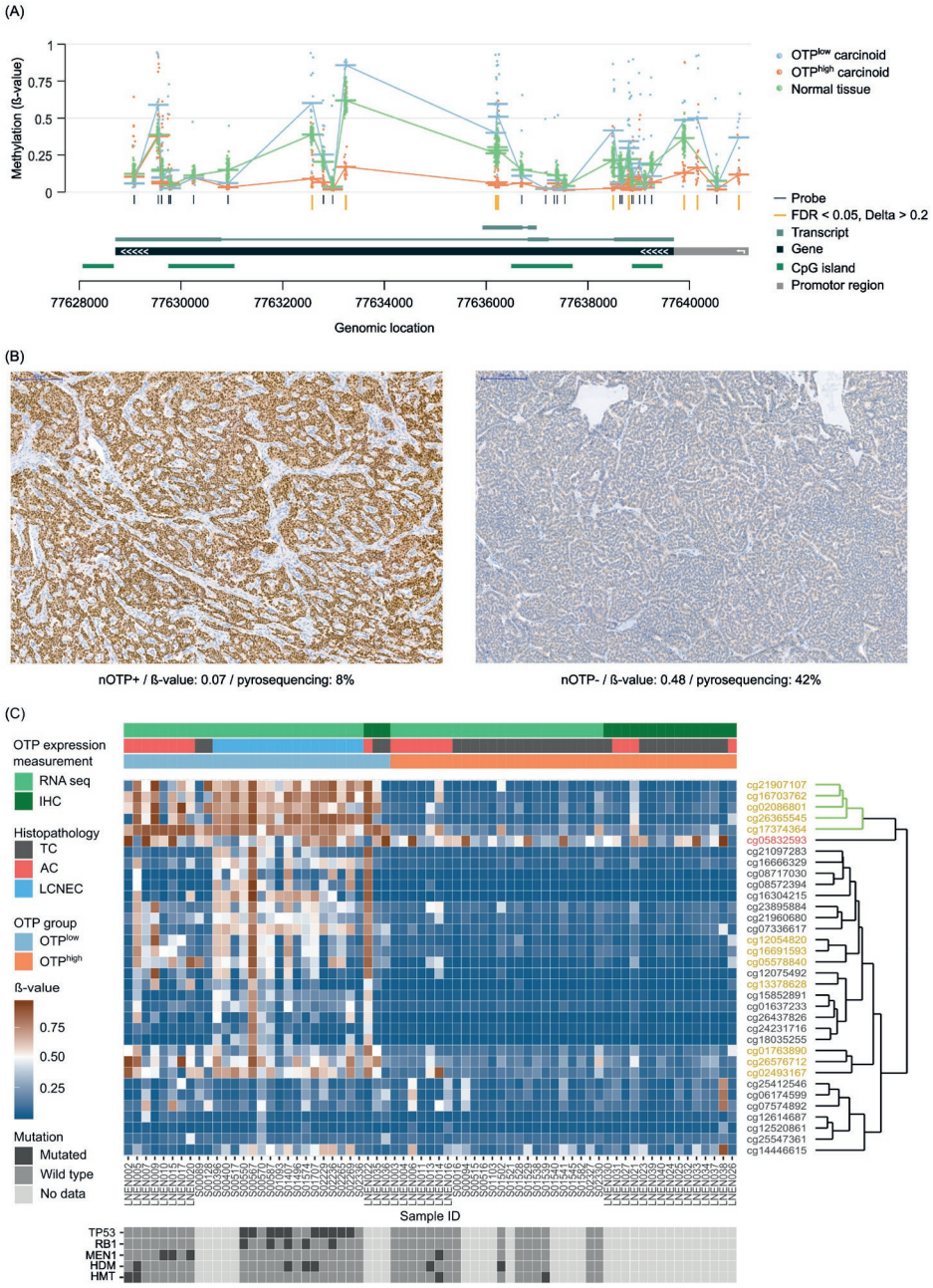

Figure 7.2 (**A**) Plot showing the DNA methylation levels at each OTP infinium probe (850K) of all carcinoid samples (OTP^{high} and OTP^{low}) combined with TCGA normal lung adenocarcinoma and squamous cell carcinoma tissues (450K). The y-axis on the right shows the β values; a horizontal bar was drawn at the median β-value for each probe. Differential DNA methylation between OTP^{high} and OTP^{low} carcinoids was calculated using the Wilcoxon rank-sum test (significant different cg-sites

are presented in yellow). (**B**) Representative images illustrating OTP IHC of a pulmonary carcinoid patient showing nuclear OTP positivity and a low methylation level (left panel) and a pulmonary carcinoid patient showing absence of OTP protein expression and a high methylation percentage (right panel). (**C**) Heatmap of the methylation level (in β values) for the OTP^{high} and OTP^{low} group (x-axis) for each cg-site (y-axis). The cg-sites which harbor a significantly different methylation level between the groups are presented in yellow. The upper green legend bar represents the OTP level measurement, the middle bar represents the histopathological diagnosis of each sample and the lower bar indicates the OTP group. Somatic mutations are represented in the lower rectangle.

Discussion

Pulmonary carcinoids are rare lung tumors with a relatively indolent course of disease although a subgroup shows a more aggressive disease course. Previously, we have shown that low *OTP* gene and protein expression is associated with a poor prognosis and others have shown that OTP expression may be utilized to identify patients at risk for disease recurrence.[11] However, thus far the mechanisms underlying the regulation of OTP expression have not been clarified. Here, we evaluated *OTP* expression and methylation levels within 208 LNEN samples, 33 other tumor subtype cohorts and normal lung tissue using publicly available transcriptomics and methylomics data and identified a unique and bimodal expression of *OTP* in lung carcinoids. To date, no mutations, or other genomic modifications (i.e., chimeric transcripts and/or genomic rearrangements) have been reported in the *OTP* gene. Therefore, we comprehensively analysed epigenomic data, revealing, for the first time, that differential *OTP* expression patterns could be explained by epigenetic modifications. Our findings were verified in 30 additional pulmonary carcinoids samples from Laddha et al.

Previously, Alcala et al. correlated gene expression and promoter methylation in pulmonary carcinoids to identify genes, which expression can be explained by methylation of CpG islands.[15] Results highlighted several top candidate genes including two homeobox genes (*HNF1A* and *HNF4A*). However, *OTP*, although methylated, was not among these top candidate genes. This might be the results of the fact that DNA methylation does not occur exclusively at CpG islands. Most of the tissue-specific DNA methylation seems to occur at CpG island shores (region of lower CpG density that lie in close proximity (~2 kb) of CpG island)[26-29] as analysed in depth here.

One of the cg sites, i.e., cg17374364, harboured a β-value above 0.5 in all OTP^{low} samples. Nevertheless, also five of the 39 OTP^{high} samples showed a β-value above 0.5 (LNEN013, LNEN014, S01502, S02330, LNEN021). Some of these samples carried mutations in lysine demethylases of histone methyltransferases, which might have impacted the methylation status of the above-mentioned cg site.

In relation to the spectrum of LNENs (low-grade to LCNEC), we and others have shown that downregulation of OTP expression is correlated with poor prognosis. Both a subgroup of carcinoids as well as LCNEC are OTP negative. It is interesting to investigate whether this observation matches with other observations in these tumors suggesting a temporal transition from low-grade to high-grade NE carcinomas.[30,31] Alcala *et al.* and Laddha *et al.* identified through different analyses and in a different dataset, three equivalent molecular groups of carcinoids (i.e., A1, A2, and B *vs.* LC1, LC2, and LC3, with different clinical features).[15,16,23] Importantly, in both the A1-A2 and LC1-LC3 groups *OTP* was generally highly expressed, while *OTP* was downregulated in the B and LC2 group. Moreover, they showed that within cluster A1 a sub cohort existed, also referred to as 'supra'-carcinoids, with molecular and clinical characteristics most similar to LCNEC; these supra-carcinoids also showed low *OTP* expression. Alcala *et al*. showed that these supra-carcinoids are, albeit their shared low *OTP* expression, vastly different from the carcinoid B cluster on the molecular level.[15] Despite these distinct genomic features based on both genome-wide expression and methylation profiles, we here observe that these supra-carcinoids showed all high methylation levels similar to the OTP^{low} carcinoids. Most remarkable is LNEN021, because this supra-carcinoid clustered as OTP^{high} based on IHC analysis, but harboured high methylation levels on cg17374364 in contrast to the other OTP^{low} samples. This suggests that despite the high genomic difference between OTP^{low} and supra-carcinoids, they share this common feature of high methylation levels on a specific CpG in the *OTP* gene. It remains to be investigated whether these clusters of carcinoids evolve or whether they are distinct entities from the beginning and progress as a result of punctuated tumor evolution.[32]

The high expression levels in pulmonary carcinoids as compared to all other cancers, the correlation of loss of expression and poor prognosis, and the simplicity of IHC to detect expression mark OTP as a highly suitable diagnostic and prognostic marker for daily clinical practice. However, different studies have reported additional genes which expression correlated with overall patient survival.[9,15,23,33,34]. It remains to be studied whether a panel of markers can further improve the prognostication of pulmonary carcinoids.

Our study implicates DNA methylation as possible regulatory mechanism of *OTP*; nevertheless, this study has several limitations. Firstly, it is a retrospective study with a rather small cohort. Albeit pulmonary carcinoid is an orphan disease, future studies should validate our findings using larger cohorts containing extensive clinical data (i.e., relapse free survival). Secondly, our study did not contain data on the methylation level of normal neuroendocrine cells of the lung. It would be very informative to investigate the methylation levels in these specific cells and in other neuroendocrine tumor types in

future studies. Furthermore, we should investigate whether methylation may occur as the result of temporal tumor evolution by analysing the methylation levels in tissue of primary carcinoid tumors at diagnosis that later developed disease recurrence as well as the corresponding metastatic lesion. Thirdly, even though we did not observe significant differential expression levels in the TET (TET1, TET2, TET3)- and DNMT family (DNMT1, DNMT3A, DNTM3B), two enzyme families that play a major role in DNA methylation, between OTP^{high} and OTP^{low} carcinoids, future studies should investigate the members of the methylation machinery in more detail (Supplementary Table S7.3). Lastly, future studies might investigate the role of miRNAs as additional regulators of OTP expression.[35]

Conclusion

We show that within the group of pulmonary carcinoid patients, two distinct groups exist which can be separated based on *OTP* expression. To our knowledge, we are the first to prove that the differential *OTP* expression within pulmonary carcinoids is associated with changes in DNA methylation. These findings arouse curiosity whether epigenetic therapies might be useful for pulmonary carcinoid patients in the future.

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Supplemental material

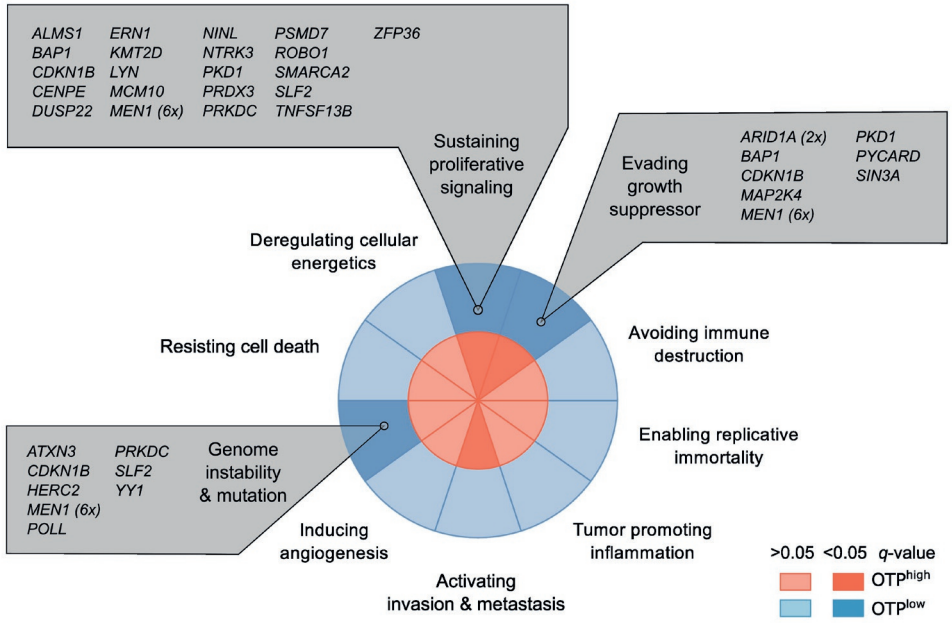

Figure S7.1 Characteristic hallmarks of cancer in the OTP^{high} and OTP^{low} group. Coloured concentric circles correspond to the groups. For each group, dark colours highlight significantly enriched hallmarks (Fisher's exact test q-value < 0.05).

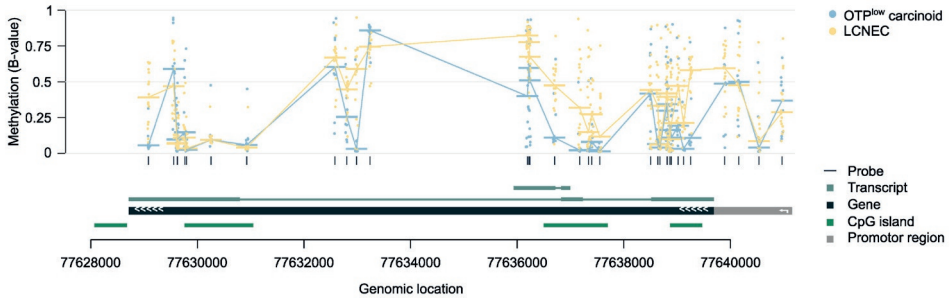

Figure S7.2 Plot showing the DNA methylation levels at each OTP infinium probe (850K) of OTP^{low} carcinoids and LCNEC samples. The y-axis on the right shows the β-values; a horizontal bar was drawn at the median β-value for each probe. Differential DNA methylation between OTP^{high} and OTP^{low} carcinoids was calculated using the Wilcoxon rank sum test (significant different cg-sites are presented in yellow).

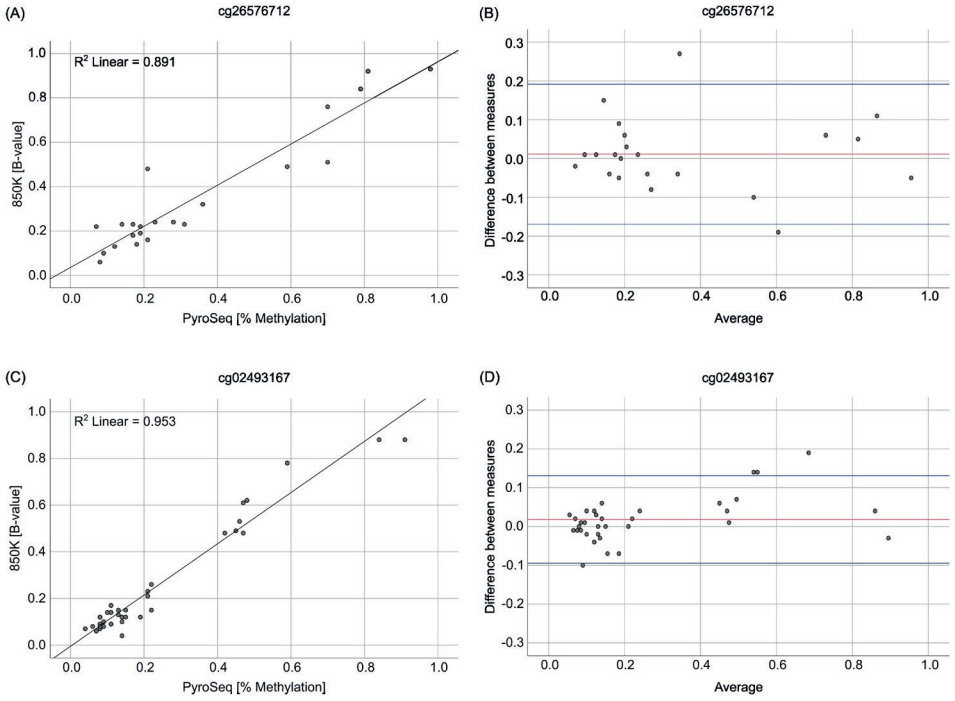

Figure S7.3 Cross-validation of 850K methylation array data and quantitative pyrosequencing. Scatter plots for the methylation values obtained by pyrosequencing (x-axis) and the β-values obtained from the 850K array (y-axis) for cg26576712 (A) and cg02493167 (C). Each dot in the diagram represents the comparison of % methylation according to pyrosequencing versus the β-value for a single cg-site. (B) and (D) represent corresponding Bland-Altman plots. The difference between both methods for every individual measurement is plotted against the mean of both methods. The mean of the differences +/- two times the standard deviation denotes the 95% range for the limits of agreement (marked by the blue lines). For the construction of these plots, the percentages pyrosequencing have been divided by a factor 100 in order to obtain data sets of the same size range.

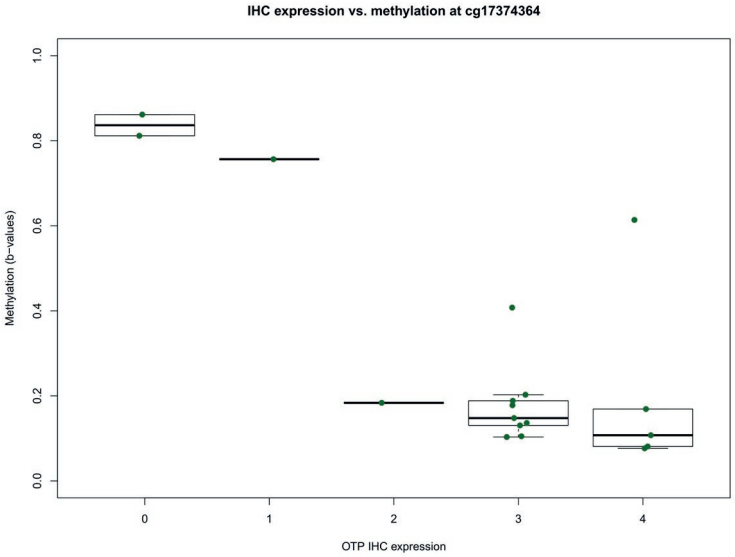

Figure S7.4 Boxplot showing the OTP IHC expression (x-axis) and the corresponding methylation level on cg17374364 (y-axis) of pulmonary carcinoids patients. Centre lines represent the median and box bounds represent the interquartile range (IQR).

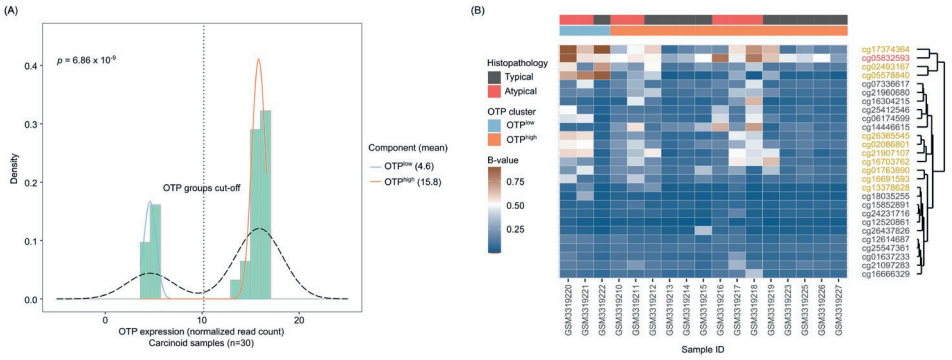

Figure S7.5 Confirmation of findings on Laddha et al data. (A) Histogram presenting the OTP expression pattern, in units of normalized read counts, in pulmonary carcinoids. Striped curve represent the distribution fit of the two Gaussian mixture distributions (component 1, in blue, and component 2, in orange). The OTP cut-off is determined as the lowest density point of the two Gaussian mixture distributions (upper panel, x = 10.2). (B) Heatmap of the methylation level (in β-values) for the OTP^{high} and OTP^{low} group (x-axis) for each cg-site (y-axis). The cgs which harbour a significantly different methylation level in the original data between the groups are presented in yellow. The upper legend bar represents the histopathological diagnosis of each sample, and the lower bar indicates the OTP group.

Table S7.1 is available at https://onlinelibrary.wiley.com/doi/full/10.1002/ijc.33939

Table S7.2 Methylation overview for each OTP cg site.

probeID	CpG_chrm	CpG_beg	CpG_end	probe_strand	geneNames	transcriptTypes	transcriptIDs	distToTSS	CGI	CGIposition	diff	p	fdr
cg17374364	chr5	77633238	77633240	+	OTP	protein_coding	ENST00000306422.4	6450	CGI:chr5:77629751-77631050	S_Shelf	0,688663282	2,89793E-07	9,85296E-06
cg02086801	chr5	77632580	77632582	+	OTP	protein_coding	ENST00000306422.4	7108	CGI:chr5:77629751-77631050	S_Shore	0,513772333	2,95894E-06	5,03019E-05
cg05578840	chr5	77638501	77638503	-	OTP	protein_coding	ENST00000306422.4	1187	CGI:chr5:77638864-77639471	N_Shore	0,391557605	1,0036E-05	0,00011279
cg16703762	chr5	77636221	77636223	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	3467;772	CGI:chr5:77636492-77637698	N_Shore	0,529909659	1,32695E-05	0,00011279
cg21907107	chr5	77636236	77636238	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	3452;757	CGI:chr5:77636492-77637698	N_Shore	0,462422971	3,7063E-05	0,000252028
cg21960680	chr5	77632802	77632804	-	OTP	protein_coding	ENST00000306422.4	6886	CGI:chr5:77629751-77631050	S_Shore	0,188260644	4,70227E-05	0,000266462
cg12054820	chr5	77638812	77638814	+	OTP	protein_coding	ENST00000306422.4	876	CGI:chr5:77638864-77639471	N_Shore	0,247700491	0,00011488	0,00048824
cg16666329	chr5	77636700	77636702	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	2988;293	CGI:chr5:77636492-77637698	Island	0,050923735	0,00011488	0,00048824
cg24231716	chr5	77638675	77638677	-	OTP	protein_coding	ENST00000306422.4	1013	CGI:chr5:77638864-77639471	N_Shore	0,011500061	0,00017386	0,000656806
cg16691593	chr5	77638808	77638810	+	OTP	protein_coding	ENST00000306422.4	880	CGI:chr5:77638864-77639471	N_Shore	0,257510494	0,000452687	0,001539136
cg12075492	chr5	77638893	77638895	-	OTP	protein_coding	ENST00000306422.4	795	CGI:chr5:77638864-77639471	Island	0,085704839	0,000907113	0,002803803
cg26576712	chr5	77640156	77640158	-	OTP	protein_coding	ENST00000306422.4	-468	CGI:chr5:77640301-77641159	N_Shore	0,334742291	0,001474237	0,004177006
cg01637233	chr5	77637398	77637400	+	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	2290;-405	CGI:chr5:77636492-77637698	Island	0,022210606	0,00200677	0,005248475
cg13378628	chr5	77638867	77638869	-	OTP	protein_coding	ENST00000306422.4	821	CGI:chr5:77638864-77639471	Island	0,147537951	0,002331262	0,005661635
cg26365545	chr5	77636190	77636192	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	3498;803	CGI:chr5:77636492-77637698	N_Shore	0,340639551	0,003594103	0,007637468
cg26437826	chr5	77640535	77640537	+	OTP	protein_coding	ENST00000306422.4	-847	CGI:chr5:77640301-77641159	Island	0,023248288	0,003594103	0,007637468
cg01763890	chr5	77640967	77640969	+	OTP	protein_coding	ENST00000306422.4	-1279	CGI:chr5:77640301-77641159	Island	0,251397999	0,007026699	0,014053398
cg12614687	chr5	77630926	77630928	-	OTP	protein_coding	ENST00000306422.4	8762	CGI:chr5:77629751-77631050	Island	0,026892783	0,00903687	0,017069644
cg21097283	chr5	77638635	77638637	-	OTP	protein_coding	ENST00000306422.4	1053	CGI:chr5:77638864-77639471	N_Shore	0,014316159	0,010213811	0,018277345
cg14446615	chr5	77629081	77629083	-	OTP	protein_coding	ENST00000306422.4	10607	CGI:chr5:77628062-77628677	S_Shore	-0,045777861	0,012964006	0,02203881
cg07574892	chr5	77629764	77629766	+	OTP	protein_coding	ENST00000306422.4	9924	CGI:chr5:77629751-77631050	Island	0,086686744	0,016317916	0,026419483
cg16304215	chr5	77632984	77632986	-	OTP	protein_coding	ENST00000306422.4	6704	CGI:chr5:77629751-77631050	S_Shore	0,014999148	0,018252253	0,028208028
cg07336617	chr5	77639254	77639256	-	OTP	protein_coding	ENST00000306422.4	434	CGI:chr5:77638864-77639471	Island	0,041775147	0,022701352	0,03355852
cg02493167	chr5	77639893	77639895	+	OTP	protein_coding	ENST00000306422.4	-205	CGI:chr5:77640301-77641159	N_Shore	0,357392754	0,025244452	0,034332454
cg23895884	chr5	77639019	77639021	-	OTP	protein_coding	ENST00000306422.4	669	CGI:chr5:77638864-77639471	Island	0,092843829	0,025244452	0,034332454
cg05832593	chr5	77629554	77629556	+	OTP	protein_coding	ENST00000306422.4	10134	CGI:chr5:77629751-77631050	N_Shore	0,213394744	0,170495974	0,222956274
cg15852891	chr5	77639120	77639122	+	OTP	protein_coding	ENST00000306422.4	568	CGI:chr5:77638864-77639471	Island	0,003056847	0,286578304	0,360876383
cg08572394	chr5	77637170	77637172	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	2518;-177	CGI:chr5:77636492-77637698	Island	0,001427176	0,467456642	0,548052615
cg18035255	chr5	77637549	77637551	+	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	2139;-556	CGI:chr5:77636492-77637698	Island	0,001620563	0,467456642	0,548052615
cg12520861	chr5	77629797	77629799	+	OTP	protein_coding	ENST00000306422.4	9891	CGI:chr5:77629751-77631050	Island	-0,000561835	0,615480827	0,697544937
cg06174599	chr5	77629620	77629622	+	OTP	protein_coding	ENST00000306422.4	10068	CGI:chr5:77629751-77631050	N_Shore	0,025705514	0,696216525	0,763592318
cg25412546	chr5	77629624	77629626	+	OTP	protein_coding	ENST00000306422.4	10064	CGI:chr5:77629751-77631050	N_Shore	0,041831023	0,752024211	0,799025724
cg25547361	chr5	77630253	77630255	-	OTP	protein_coding	ENST00000306422.4	9435	CGI:chr5:77629751-77631050	Island	-0,000713059	0,896531195	0,923698807
cg08717030	chr5	77637337	77637339	-	OTP;OTP	protein_coding;protein_coding	ENST00000306422.4;ENST00000515716.1	2351;-344	CGI:chr5:77636492-77637698	Island	-0,00142891	0,95555793	0,95555793

CpG_chrm = CpG chromosome; CpG_beg = Genomic location where the CpG starts; CpG_end = Genomic location where the CpG ends; probe_strand = Strand to which the probe binds; geneNames = Target Gene; transcriptTypes = Type of transcript that is targeted; transcriptIDs = ID of the targeted transcript; distToTSS = Distance to the transcription start site; CGI = CpG Island; CGIposition = Genomic position of the CpG Island; diff = Difference in DNA methylation between the OTP^{high} and OTP^{low} group; *p = P*-values determined following the wilcoxon rank sum test; fdr= false discovery rate.

Table S7.3 Differential gene expression analysis between OTP^{high} and OTP^{low} carcinoids for genes of the TET and DNMT family.

Gene	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
TET1	185.565693667492	0.0991985890219099	0.227478832748159	0	1	1
TET2	1716.15427524981	-0.0699377115530578	0.140823018419193	0	1	1
TET3	495.352196677641	-0.304670478334423	0.208328313072144	0	1	1
DNMT1	1752.40021250761	-0.295085767885599	0.147021273576311	0	1	1
DNMT3A	1040.28737278851	-0.100639524326014	0.163376193987811	0	1	1
DNMT3B	179.543687984937	0.0208769843912612	0.248226302159636	0	1	1

Gene = Gene HUGO symbol; baseMean = Base mean in the comparison OTP^{high} vs. OTP^{low}; log2FoldChange = Log2 fold change in the comparison OTP^{high} vs. OTP^{low}; lfcSE = Standard error of the log2 fold change estimate in the comparison of OTP^{high} vs. OTP^{low}; stat = Wald statistic in the comparison of OTP^{high} vs. OTP^{low}; pvalue = p-value in the comparison OTP^{high} vs. OTP^{low}; padj = p-value adjusted by the Benjamini-Hochberg procedure, in the comparison OTP^{high} vs. OTP^{low}.

Table S7.4 TCGA abbreviations.

Tumor types Abbreviation	Full name
Typical	Typical carcinoid
Atypical	Atypical carcinoid
LCNEC	Large cell neuroendocrine carcinoma
SCLC	small cell lung cancer
LGG	Brain lower grade glioma
GBM	Glioblastoma multiforme
UCS	Uterine Carcinosarcoma
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
STAD	Stomach adenocarcinoma
ESCA	Esophageal carcinoma
PRAD	Prostate adenocarcinoma
LAML	Acute Myeloid Leukemia
LUSC	Lung squamous cell carcinoma
BLCA	Bladder Urothelial Carcinoma
SARC	Sarcoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
SKCM	Skin Cutaneous Melanoma
CHOL	Cholangiocarcinoma
UCEC	Uterine Corpus Endometrial Carcinoma
TGCT	Testicular Germ Cell Tumors
PCPG	Pheochromocytoma and Paraganglioma
ACC	Adrenocortical carcinoma
LUAD	Lung adenocarcinoma
KIRC	Kidney renal clear cell carcinoma
BRCA	Breast Invasive Carcinoma
HNSC	Head and Neck squamous cell carcinoma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
MESO	Mesothelioma
THCA	Thyroid carcinoma
THYM	Thymoma
COAD	Colon adenocarcinoma
READ	Rectum adenocarcinoma
LIHC	Liver hepatocellular carcinoma
KICH	Kidney Chromophobe
KIRP	Kidney renal papillary cell carcinoma
UVM	Uterine Corpus Endometrial Carcinoma

CHAPTER 8

Development and verification of new monoclonal Orthopedia Homeobox (OTP) specific antibodies for pulmonary carcinoid diagnostics

L. Moonen, J. L. Derks, L. M. V. Lap, B. J. C. A. Marijnissen, L. M. Hillen, M. A. den Bakker, J. H. von der Thüsen, R. J. van Suylen, W. Timens, M. Bintanel, E. Kuteeva, A-M. C. Dingemans, E. J. M. Speel

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Abstract

Background

Orthopedia Homeobox (OTP) has shown to be a useful prognostic marker to predict outcome in pulmonary carcinoids, which is also supported by the World Health Organization. However, the discontinuation of the initially used polyclonal antibody and absence of a reliable routinely applicable monoclonal OTP antibody hampers implementation in routine diagnostics. Here, new monoclonal antibodies directed against OTP were developed and verified on formalin-fixed paraffin-embedded tissue of pulmonary neuroendocrine tumors for clinical diagnostics.

Methods

OTP specific monoclonal antibodies were produced from mice immunised with a recombinant human OTP protein fragment. Enzyme-linked immunosorbent assay (ELISA) positive hybridomas were evaluated using immunohistochemistry (IHC). Following epitope-mapping and isotyping, purified monoclonal antibodies were validated for IHC in formalin-fixed paraffin-embedded tissues, the optimal dilution was determined, and results were cross validated with the OTP polyclonal antibody (HPA039365, Atlas Antibodies). Staining protocols were optimized on two automated staining platforms and performance was harmonized using a tissue micro array.

Results

Two clones (CL11222 and CL11225) were selected for purified monoclonal antibody production. Intratumor heterogeneity assessment revealed similar performance for both clones. While clone CL11225 displayed a unique epitope compared to those present in the polyclonal antibody, this clone performed most similar to the polyclonal antibody. Cross-platform assessment revealed an excellent agreement for clone CL11225 while clone CL11222 showed somewhat discordant results on Dako.

Conclusions

New monoclonal OTP specific antibodies have been developed and verified on different automated immunohistochemical staining platforms. The OTP specific monoclonal antibodies showed excellent agreement with the often-used polyclonal antibody allowing application in routine diagnostics.

Introduction

The use of cancer biomarkers is nowadays indispensable in clinical practice. A cancer biomarker, as defined by the World Health Organization (WHO), is any substance, structure or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease.[1] In clinical terms, a cancer biomarker may estimate the risk of developing cancer, cancer progression or may predict therapy response in a specific tissue. Therefore, cancer biomarkers can be subdivided into either diagnostic, prognostic or predictive biomarkers.[1]

Pulmonary carcinoids are rare well-differentiated neuroendocrine neoplasms which can be subdivided into typical and atypical carcinoid, based on the mitotic index and the presence of necrosis.[2] Nevertheless, due to high morphological similarities, the existing histology-based grading is subject to considerable interobserver variation.[3] Moreover, the current classification is imprecise on (preoperative) biopsy specimens.[4] In 57% of the patients the preoperative biopsy specimen diagnosis is discordant with the paired resection specimen diagnosis. Therefore, the current WHO classification advises not to separate typical vs. atypical carcinoid on a (limited) biopsy specimen.[2] Together these data indicate the need for additional molecular markers that help to decrease inter-observer variation and improve prognostic prediction particularly on limited tissue specimen.

By genomic profiling, we have recently identified orthopedia homeobox (OTP) as a biomarker for pulmonary carcinoids with a favourable prognosis, i.e., prognostic biomarker.[5] The prognostic value of nuclear OTP protein expression has since been validated in independent series, confirming that loss of expression is associated with poorer prognosis.[6,7] OTP encodes a member of the homeodomain protein family, which are helix-turn-helix transcription factors that play key roles in the specification of cell fates such as the formation of somatostatin neurons in the arcuate nucleus of the hypothalamus.[8] While the role of OTP in pulmonary physiology has not yet been unravelled, various neuropeptides which are under control of OTP in the hypothalamus (such as neuropeptide Y (NPY), Agouti-related protein (AgRP), and somatostatin) are expressed by pulmonary neuroendocrine cells.[9,10] Further, OTP has been proven to be a highly specific marker for low grade neuroendocrine pulmonary neoplasms. Consequently, an increasing number of studies are evaluating the diagnostic utility of OTP expression in pulmonary carcinoids.[11-15] Moreover, in the current WHO 2021 criteria OTP is reported as a promising molecular marker for the prognostication of pulmonary carcinoids.[2] Nevertheless, due to the discontinuation of the initially used rabbit polyclonal antibodies (pAb) (HPA039365, Atlas Antibodies, Stockholm, Sweden),

variations in quality and staining conditions required for the use of OTP pAbs, and the unavailability of a similarly performing monoclonal antibody (mAb), implementation of this marker in routine diagnostics is hampered.

Here, two new mAbs directed against OTP (CL11222, AMAb91695 and CL11225, AMAb91696, Atlas antibodies, Stockholm, Sweden) were developed and verified on formalin-fixed paraffin-embedded (FFPE) tissue of pulmonary neuroendocrine tumors for clinical diagnostics. First, we generated and validated the antibodies using normal tissues and mapped the binding epitopes. Second, immunohistochemical staining protocols were manually optimized and cross-validated with the reference rabbit pAb (HPA039365, Atlas Antibodies, Stockholm, Sweden). Third, staining protocols were further optimized for the Autostainer 480S (ThermoFisher Scientific, Waltham, MA, USA) and the Dako Autostainer Link 48 (Agilent Technologies, Santa Clara, CA) and performance was harmonized using a pulmonary neuroendocrine tumor tissue micro array (TMA). We present the following article in accordance with the STARD 2015 reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-418/rc).

Methods

Development of monoclonal OTP antibodies and IHC on normal tissues

Anti-OTP mAbs were developed by Atlas Antibodies using a protocol established by a commercial service provider. Briefly, mice were immunised using a recombinant protein fragment corresponding to a.a. 27-120 of human OTP coupled to HIS-APP. B-cells from immunized mice were then fused with a myeloma cell line (SP2/0) to produce hybridomas. Crude supernatants from ELISA positive hybridomas were evaluated using immunohistochemistry (IHC) on relevant human tissues (see below). In addition, the Ig isotype of mAbs was defined, and antibodies were epitope-mapped using linear peptides (see below). Following screening results, two clones were selected for subcloning, culturing, and production of purified mAbs: CL11222 (AMAb91695) and CL11225 (AMAb91696). Final concentration was set at 1 mg/ml for both antibodies. Purified mAbs were validated for IHC in FFPE tissues to confirm specificity and selectivity, as well as to define optimal dilution for IHC application. Isotype and epitope were confirmed using purified monoclonals. pAb HPA039365 (Atlas Antibodies, Stockholm, Sweden) was used as reference in epitope-mapping.

The antibody isotype was defined using the Milliplex Isotyping Kit (Milliplex®MAP mouse Immunoglobulin Isotyping Magnetic Bead Panel, Merck/Millipore). The epitopes were defined using unpurified synthetic N-terminally biotinylated peptides (15 a.a. long, overlapping by 10 a.a.) (Pepscan Presto, Lelystad, The Netherlands). Peptides were

coupled to Luminex neutravidin beads (Mspher, Lx100, LumAv, 5.6um, 1mL vial, 2.5 million bead/mL conc., PBSTBN). A master mix containing magnetic beads for measuring mouse IgG subclasses (IgG1, IgG2a, IgG2b, IgG3, IgGM and IgGA) and 1% bovine serum albumin (BSA) diluted in 1x phosphate buffered saline (PBS) was prepared. Antibody (2 ul) was mixed with 50 ul of master mix and 17 ul of epitope-mapping beads in 96-well round bottomed assay plate (Costar, 3792) and incubated for 1 hour at room temperature on a tabletop shaker at 600 rpm. Then, the secondary anti-Mouse k light chain-PE diluted 1:500 was added and incubated for 1 hour at room temperature on a tabletop shaker at 600 rpm. For the pAb, the same method was followed, but F(ab')2 Fragment Donkey anti-Rabbit IgG (H+L), PE (Jackson Antibodies 711-116-152) diluted 1:100 was used for the detection. The plate was read in the BIO-RAD Bio-Plex 200 flow cytometer system and Bio-Plex Manager 6.2 software (Bio-Rad Laboratories, Solna, Sweden) was used to analyse the results.

Specificity of OTP mAbs was evaluated in immunohistochemical experiments. Tissue sections (4 µm) were cut from a TMA constructed from commercially obtained normal tissues, including cervix, skin, oral mucosa, kidney, liver, pancreas, cerebellum, cerebral cortex, testis and prostate, skeletal muscle and heart muscle, colon, rectum, small intestine, duodenum, stomach and salivary gland, fallopian tube, endometrium, placenta, breast, tonsil and lymph node, adipose tissue, urothelium, and lung samples (Asterand®, BioIVT, West Sussex, UK). In addition, sections were taken from human hypothalamus (Asterand®, BioIVT, West Sussex, UK). Prior to immunostaining, sections were incubated at 50°C overnight and deparaffinized in xylene and graded ethanol. Antigen retrieval was then performed using citrate buffer pH 6.1 (S1999, Target Retrieval Solution, Citrate pH 6.1, DAKO, Agilent, Santa Clara, CA, USA) in decloaking chamber (Biocare Medical, Walnut Creek, CA, USA). Following antigen retrieval, sections were stained with anti-OTP mAbs CL11222 and CL11225 diluted 1:200 – 1:1000 (30 min at room temperature) in Autostainer 480S (ThermoFisher Scientific, Waltham, MA, USA) using a commercial kit (UltraVision LP HRP Polymer®, Primary Antibody Enhancer, Ultra V Block and DAB Quanto Chromogen ad Substrate, ThermoFisher Scientific, Waltham, MA, USA). Slides were counterstained with Mayers hematoxylin (Histolab, Sweden) and mounted using Pertex (Histolab, Sweden) for automated coverslipping. Slides were examined under a microscope (AxioScope A.1, Zeiss), and images were taken using an automated system (VSlide, MetaSystems Hard & Software GmbH, Germany).

Tumor samples

This study was conducted using FFPE resection specimen of a randomly selected retrospective series of patients with a pulmonary neuroendocrine neoplasm (PNENs) diagnosed between 2003-2012 in medical centres of the Netherlands. Patient material

consisted of typical carcinoids (TC, n=38), atypical carcinoids (AC, n=14), carcinoid not otherwise specified (carcinoid NOS, n=19), large cell neuroendocrine carcinomas (LCNEC, n=12), and small cell lung cancer (SCLC, n=3). All patients were diagnosed following the World Health Organization (WHO) criteria for pulmonary neuroendocrine tumors.[2]

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the medical ethics committee of the Maastricht University Medical Centre (METC azM/UM 16-4-106). Informed consent was not required from the patients because all biological samples are from archival materials that are exempt from consent in compliance with applicable laws and regulations (Dutch laws: Medical Treatment Agreement Act (WGBO) art 458 / GDPR art 9/ uAVG art 24).

Tissue microarray development

FFPE tissue blocks were collected from all patients (n=86). Sections of the FFPE blocks were stained with haematoxylin and eosin and representative tumor regions were marked by a pathologist (LMH). To construct a TMA, three cores of every tumor tissue, 1.0-mm in diameter, were sampled into a donor block using the fully automated TMA Grand Master (Sysmex, Norderstedt, Germany). The cores were taken from both central and peripheral parts of the tumor to allow intratumor heterogeneity analysis. Subsequently, serial 4 um sections were cut for further analysis.

Immunohistochemistry

Manual IHC detection using polyclonal OTP antibody

IHC on FFPE tissue sections was performed using a rabbit anti-OTP pAb (HPA039365; Atlas Antibodies, Stockholm, Sweden). Antibody was diluted 1:3000 and incubated overnight. Antibodies were detected by Bright Vision Poly-HRP-anti-mouse/rabbit/rat immunoglobulin G (IgG; Immunologic, Duiven, The Netherlands) followed by peroxidase-3,3'-diaminobenzidine (DAB) visualization. Tissue sections were counterstained with haematoxylin, dehydrated, and mounted. Slides were scanned at 20x magnification using the 3DHistech P1000 scanner (3DHistech, Budapest, Hungary). The pAb staining was used as a reference as this antibody has been widely used to target OTP.

Manual and automated IHC using the new monoclonal OTP antibodies

Staining protocols for OTP mAbs CL11222 and CL11225 were first manually optimized with the manual pAb staining protocols as starting point. The optimal dilution of the antibody was determined by serial dilution experiments (1:50-1:500). Next, staining protocols were further optimized on two different automated staining platforms. For

staining on the Dako Autostainer Link 48, all reagents of the Dako Envision FLEX Visualization kit K8002 were used. Epredia UltraVision LP Detection System and DAB Quanto Detection System kits were used for the Thermo Fisher 480S autostainer. Optimized staining protocols are presented in Table 8.1. Subsequently, serial sections (4 µm) obtained from the PNEN TMAs were stained on the different platforms using both mAbs for further analysis. Slides were scanned as described above.

Table 8.1 Detailed IHC staining protocol for automated staining platforms.

Protocol step	Automated staining platform	
	Dako Link48	Thermofisher 480S
Antigen retrieval	Citrate buffer Dako (pH 6.1, 20min, 95°C)	Citrate buffer Dako (pH 6.1, 10min, 95°C)
Peroxidase blocking	H_2O_2 (0.3% diluted in MilliQ)	N/A
Additional blocking step	N/A	Ultra V block (RTU, 5min, RT)
Primary antibody	Dako CL11222/CL11225 (1:200, 60min, RT)*	Dako CL11222/CL11225 (1:200, 30min, RT)
Amplifier	FLEX+ Mouse linker Dako (RTU, 20min, RT)	Primary Antibody Enhancer (RTU, 20min, RT)
Secundairy antibody	FLEX HRP Dako (RTU, 30min, RT)	UltraVision LP HRP polymer (RTU, 30min, RT)
Substrate + chromogen	FLEX/DAB+ Sub-Chromogen (1:50, 10min, RT)	DAB quanto chromogen and substrate (5min, RT)
Counterstain	Mayers Hematoxylin (1 min, RT)	Mayers Hematoxylin (5min, RT)

*Primary antibody was diluted in PBS/0.1% Tween/1% BSA/0.02% Sodiumazide. Min, minutes; RTU, ready to use; RT, room temperature; HRP, horseradish peroxidase; N/A, not applicable.

Pathological assessment of OTP staining

Slides were assessed by an experienced pulmonary neuroendocrine tumor pathologist and a researcher (L.M.H., L.M.) who are familiar with the OTP staining pattern and intensity. Raters were blinded for diagnosis, reference outcome, and each other's evaluation. Protein expression was assessed for percentage of nuclear positive tumor cells (0%-100%) and staining intensity (0, 1, 2 or 3) (Figure 8.1). H-scores were calculated by multiplying percentage of positive tumor cells by intensity. The mean H-score was calculated for each patient as the mean of the three different TMA cores. Positive staining was defined as a H-score ≥50 (Supplementary Figure S8.1).

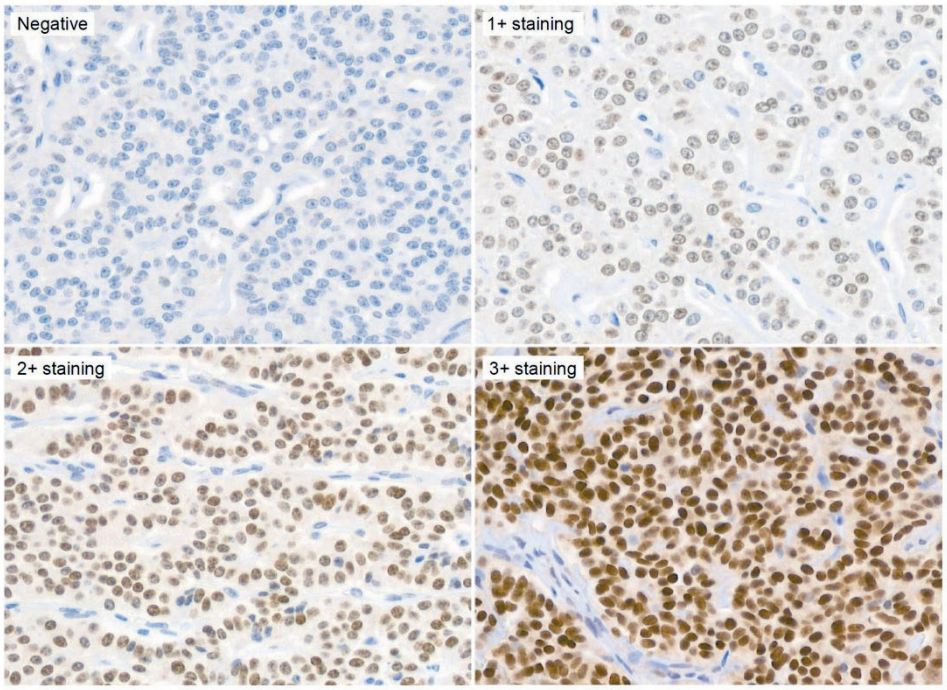

Figure 8.1 Representative immunohistochemical images showing the different OTP intensities (0,1+,2+,3+) (magnification 20x). Abbreviations: OTP, Orthopedia Homeobox.

Statistical analysis

Statistical analysis was conducted using SPSS for Mac version 26 (SPSS Inc., v26, Chicago, IL, USA). To assess the immunostaining reliability, the intraclass correlation coefficient (ICC) was calculated for continuous data and Cohen's kappa for categorical data. Two-way mixed ICC with absolute agreement definition were calculated to evaluate concordance between the H-scores between the two raters, the different cores of each patient, and the different platforms. Two-sided *p*-values <0.05 were considered significant.

Results

Monoclonal antibody isotyping and characterisation

OTP immunization of mice resulted in the identification of two mAbs. Isotyping experiments have shown that both mAbs CL11222 and CL11225 were of IgG1 isotype. Antibodies however displayed different binding sites, CL11225 epitope was located

closer to the N-terminal of the OTP protein as compared to CL11222 (Figure 8.2A). Both clones were used in further application testing and validation. In addition, the original rabbit polyclonal HPA039365 was also epitope mapped, showing signals at three different binding sites. The epitope of CL11222 corresponded to one of the epitopes of HPA039365 rabbit pAb, while CL11225 recognised a unique epitope (Figure 8.2A).

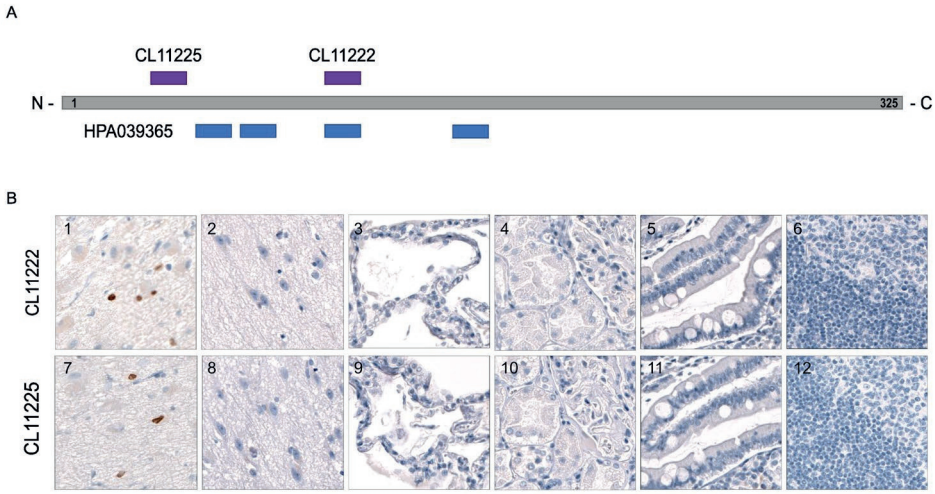

Figure 8.2 Overview of the mAb isotyping and characterisation. **A**. Linear representation of OTP protein (in grey) showing binding sites of mAb CL11222 and CL11225 (in purple), as well as pAb HPA039365 (in blue). **B.** Representative images illustrating OTP IHC of both CL11222 and CL11225 in [1,7] human hypothalamus, [2,8] cerebral cortex, [3,9] lung, [4, 10] kidney, [5,11] small intestine, [6,12] tonsil (magnification 200x). Abbreviations: OTP, Orthopedia Homeobox; mAb, monoclonal antibody; pAb, polyclonal antibody; IHC, immunohistochemistry.

According to the publicly available RNA expression profiles of the Allen Mouse brain atlas (http://portal.brain-map.org/), OTP protein expression is mainly observed in the central nervous system, both during embryonal development and in adult hypothalamus, hindbrain, and spinal cord. OTP expression in human adult tissues is mainly restricted to the hypothalamus (RNA expression level: 12.7 protein-coding transcripts per million (pTPM)), with other brain and peripheral tissues being mainly negative (RNA expression level: 0-0.3 pTPM) (Human Protein Atlas, www.proteinatlas.org). In agreement with these data, staining results of the hypothalamic tissue sections and normal tissue TMA showed that both CL11222 and CL11225 displayed moderate nuclear immunoreactivity in a subset of neurons in human hypothalamus, while no positivity was observed in e.g., cerebral cortex, lung, kidney, or any other normal peripheral tissue tested (Figure 8.2B). Titration experiments on the Thermofisher 480S autostainer revealed an optimal staining intensity at a 1:200 dilution.

Monoclonal antibody optimization for lung neuroendocrine neoplasia

Immunostaining was further optimized on a PNEN TMA containing different histological subtypes (i.e., TC, AC, carcinoid not otherwise specified (NOS), LCNEC, SCLC) using both mAbs, with the pAb HPA039365 used as a reference. Serial dilution experiments on both ThermoFisher and Dako Link48 again revealed an optimal staining intensity at 1:200 dilution. All staining protocols used resulted in a characteristic strong nuclear immunostaining pattern for OTP with an accompanying cytoplasmic component of low intensity. The surrounding normal lung tissue remained negative. Results showed similar staining patterns for the mAbs with varying positivity with the highest H-scores observed in pulmonary carcinoids (ranging from 0 to 300) while LCNEC and SCLC remained negative (Figure 8.3).

Table 8.2 summarizes the results of OTP antibody staining on the PNEN TMA. Immunostaining with the pAb reference showed nuclear positivity in 73.7% (n=28/38) of TC, 64.3% (n=9/14) of AC, and 89.5% (n=17/19) of carcinoid NOS, whereas all SCLC cases were negative (Table 8.2). In the LCNEC group, all cases were negative except for one case displaying nuclear positivity (H-score of 166.67). This case turned out to be negative when using mAb CL11222 on the DAKO platform. While positive and negative cases corresponded to a large degree when comparing pAb with both mAbs, CL11222 showed a lower number of positive cases on the DAKO platform (Table 8.2).

To investigate whether the two mAbs were specific for pulmonary carcinoids, we performed immunostaining on n=40 neuroendocrine neoplasms of non-pulmonary origin (i.e., gastroenteropancreatic neuroendocrine tumors (NETs), insulinomas, head and neck NETs, breast NETs, paragangliomas, and merkel cell carcinomas). All cases stained negative for both mAbs.

Table 8.2 Overview of the proportion of positive pulmonary neuroendocrine tumors per subtype for pAb, CL11222 and CL11225 mAb on the different staining platforms.

	TC		AC		Carcinoid NOS		LCNEC		SCLC	
	n/total	%	n/total	%	n/total	%	n/total	%	n/total	%
pAb HPA039365	28/38	73.7	9/14	64.3	17/19	89.5	1/12	8.3	0/3	0.0
Dako CL11222	25/38	65.8	8/14	57.1	17/19	89.5	0/12	0.0	0/3	0.0
ThermoFisher CL11222	28/38	73.7	9/14	64.3	17/19	89.5	1/12	8.3	0/3	0.0
Dako CL11225	28/38	73.7	10/14	71.4	18/19	94.7	1/12	8.3	0/3	0.0
ThermoFisher CL11225	28/38	73.7	9/14	64.3	17/19	89.5	1/12	8.3	0/3	0.0

Abbreviations: pAb, polyclonal antibody; mAb, monoclonal antibody; TC, typical carcinoid; AC, atypical carcinoid; NOS, not otherwise specified; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer.

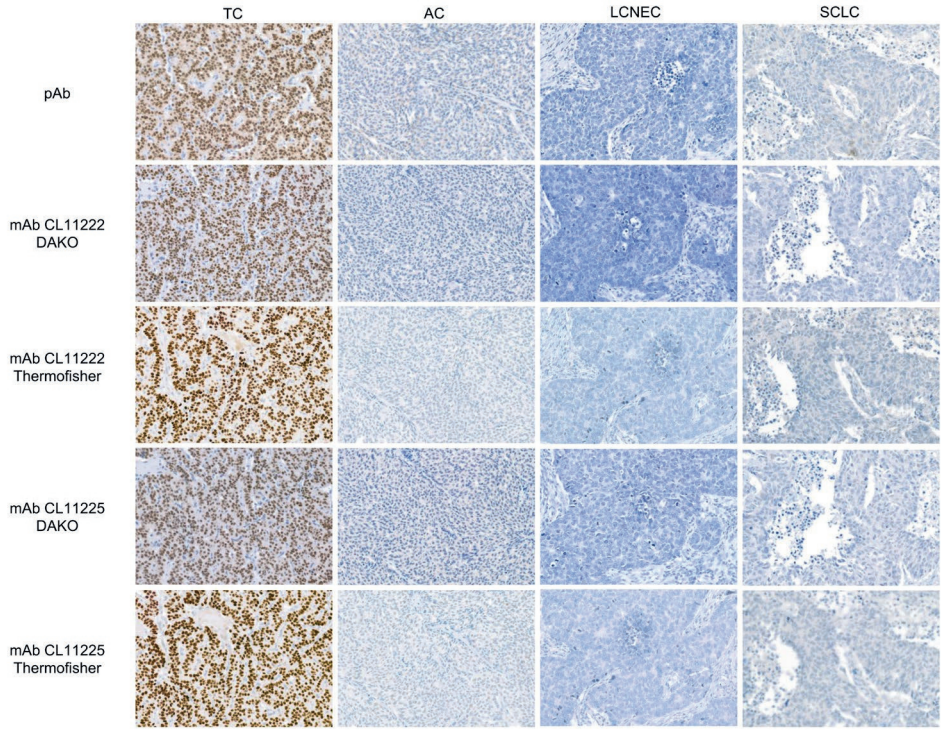

Figure 8.3 Representative images illustrating OTP immunohistochemical staining of pAb, CL11222 and CL11225 mAb in TC, AC, LCNEC, and SCLC (magnification 200x). Abbreviations: TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; pAb, polyclonal antibody; mAb, monoclonal antibody; OTP, Orthopedia Homeobox.

Interrater variability

Interrater variability was assessed for both clones on the different autostainers. Results showed, following the calculation of kappa scores, substantial agreement on the Dako platform while almost perfect agreement was reached on the ThermoFisher platform (Table 8.3). A better ICC was observed for CL11225 as compared to CL11222 on the Dako platform while ICC was comparable for both clones on the ThermoFisher platform.

Table 8.3 ICC and Cohen's kappa for the H-score between the two raters for both clones on the different platforms.

	ICC	95% CI	Kappa
Dako CL11222	0.868	0.805 - 0.912	0.767
ThermoFisher CL11222	0.977	0.954 - 0.988	1.000
Dako CL11225	0.933	0.899 - 0.956	0.789
ThermoFisher CL11225	0.978	0.966 - 0.986	0.975

Abbreviations: ICC, intraclass correlation coefficient; CI, confidence interval

Intratumor heterogeneity

Different TMA cores from the same patient were compared to assess the intratumor heterogeneity. Agreement between different cores was substantial to almost perfect (kappa ranging from 0.980 to 0.992) for both clones on both platforms scored by the individual raters (Table 8.4).

Table 8.4 ICC of the H-scores between tumor cores of the same patient scored by the raters.

	Rater 1		Rater 2	
	ICC	95% CI	ICC	95% CI
Dako CL11222	0.980	0.971 - 0.986	0.986	0.979 - 0.991
ThermoFisher CL11222	0.989	0.985 - 0.993	0.990	0.986 - 0.993
Dako CL11225	0.985	0.978 - 0.990	0.983	0.976 - 0.989
ThermoFisher CL11225	0.987	0.981 - 0.991	0.992	0.989 - 0.995

Abbreviations: ICC, intraclass correlation coefficient; CI, confidence interval.

Cross-platform harmonization

Almost perfect agreement was observed between the rabbit pAb and the two mAbs on both platforms with an overall agreement kappa of 0.900 (95% CI: 0.898-0.902). Inter-platform agreement showed that CL11225 was preferred over CL11222 since it performed best on both platforms (Table 8.5).

Table 8.5 Overview of the interplatform agreement following cohen's kappa of the H-scores for the pAb, CL11222 and CL11225 mAb on both Dako and ThermoFisher platform.

	pAb HPA039365	Dako CL11222	ThermoFisher CL11222	Dako CL11225	ThermoFisher CL11225
pAb HPA039365	-	0.878	1.000	0.898	0.975
Dako CL11222	0.878	-	0.878	0.828	0.903
Thermo Fisher CL11222	1.000	0.878	-	0.898	0.975
Dako CL11225	0.898	0.828	0.898	-	0.873
ThermoFisher CL11225	0.975	0.903	0.975	0.873	-

Abbreviations: pAb, polyclonal antibody; mAb, monoclonal antibody.

Stability of the antibody

Testing with different antigen solutions buffers and blocking buffers, however, revealed that TRIS-holding solutions resulted in a remarkable reduction of the staining intensity compared with the original staining intensity. Based on these findings, TRIS-holding solutions should be avoided to rule out false-negative staining. Further, this study cohort ranged from 2003 to 2012 indicating that the OTP staining is also applicable on older FFPE tissue.

Discussion

The current histopathological classification of pulmonary carcinoids is subject to high-interobserver variation and requires additional (molecular) analyses to improve prediction of prognosis.[3] An interesting prognostic IHC biomarker is nuclear OTP expression, which has been associated with a favourable prognosis. However, the limited availability of the current rabbit polyclonal OTP specific antibody HPA039365, variations in quality of new batches, and the unavailability of a similarly performing mAb, hampers implementation of this marker in routine diagnostics. A systematic comparison of two newly developed OTP mAbs (CL11222 and CL11225) on two different automated platforms revealed adequate immunostaining and high concordance with the initially used rabbit pAb (HPA039365, Atlas Antibodies, Stockholm, Sweden).

Several studies have investigated OTP IHC expression in pulmonary carcinoids using different primary antibodies and immunostaining protocols.[5,6,11-14] Data suggest that considerable differences exist between the staining conditions of the antibodies that are currently being used in laboratories. To implement OTP IHC in routine diagnostics, standardized assays using mAbs are required. Our results show an almost perfect agreement utilizing two commonly used automated immunostaining platforms (kappa 0.900). Inter-platform analyses showed that mAb CL11222 showed some false negativity (n=5) when used on the DAKO link48 autostainer. The finding that mAb CL11225 performed better as compared to CL11222 on the DAKO link48 platform raised the question whether this might be the result of the avidity of the different mAbs for OTP. However, this difference was not observed on the ThermoFisher 480S platform, thereby negating differences in avidity as the underlying cause. Several studies, mainly on PD-L1 IHC, have shown that specific antibodies can be used on alternative platforms to overcome implementation barriers of IHC assays.[16-19] However, these studies observed both similarities and differences between different IHC assays. Every automated staining platform has its own IHC reagents and detection system which can influence the identification of an antibody clone. It would be interesting to investigate whether this

staining difference is clone related or platform related by establishing optimized staining protocols for other commonly used platforms such as Dako Omnis, Leica Bond-III and Ventana BenchMark Ultra in future studies. All such assays should be validated by direct comparison either with the protocols provided in this study or with externally validated reference samples.

TMAs were used in the analysis for an assay-to-assay comparison between staining protocols as well as for intratumor heterogeneity assessment. The use of TMAs is often considered as a study limitation due to tumor heterogeneity. However, serial sections of TMAs were used to minimize variability. Our results showed that the intratumor heterogeneity of OTP expression was very low with a kappa ranging from 0.980 to 0.992, thus rather indicating that OTP is homogeneously expressed through the tumor resulting in a homogeneous staining pattern. These findings are in line with previous data on whole tissue sections showing OTP staining to be highly consistent.[5] As we recently showed that preoperative biopsy diagnosis is imprecise, we may advocate that the new OTP mAbs can be used, in a molecular marker panel (Ki-67, OTP, CD44), to improve current preoperative pulmonary carcinoid classification and prognostication on biopsies.[20]

In conclusion, new monoclonal OTP specific antibodies have been developed and verified on two different automated staining platforms for IHC. Comprehensive analysis showed adequate concordance and good reproducibility. The excellent performance on FFPE material may now allow application in routine diagnostics and may improve current carcinoid classification and prognostication in both pre- and post-operative settings.

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Supplemental figure

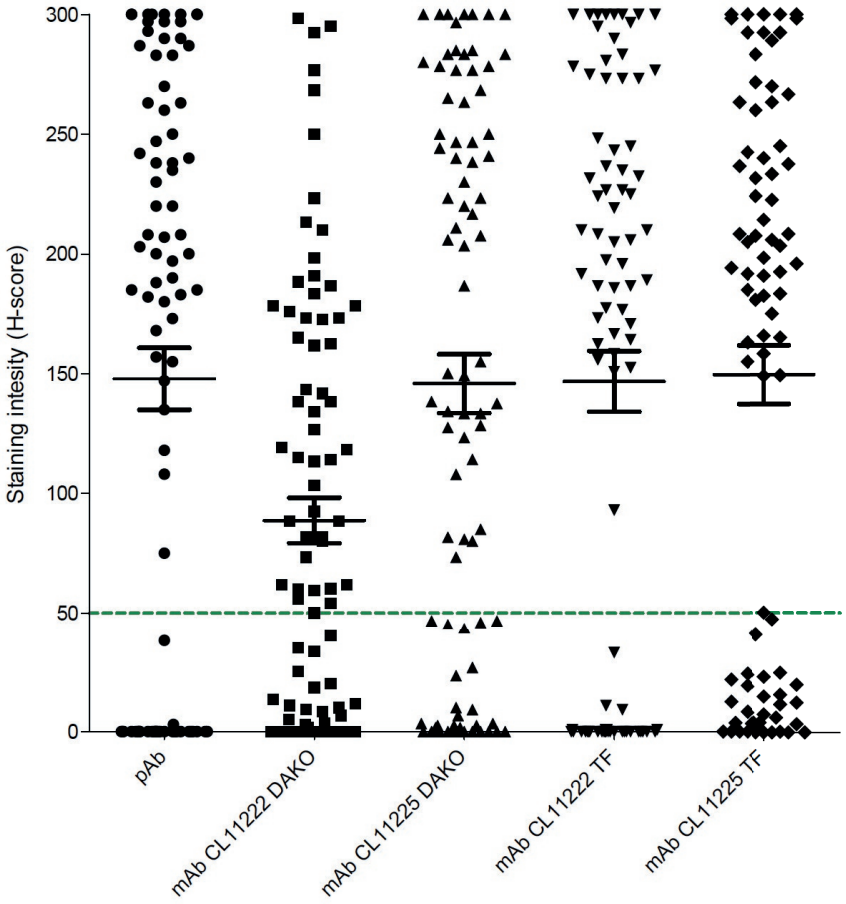

Figure S8.1 Column scatterplot showing the Orthopedia Homeobox (OTP) staining intensity (H-score) of the pAb, CL11222 and CL11225 mAb using different autostainers platforms. Every dot represents an individual tumor sample. Abbreviations: pAb, polyclonal antibody; mAb, monoclonal antibody.

CHAPTER 9

Druggable growth dependencies and tumor evolution analysis in patient-derived organoids of neuroendocrine cancer

T. L. [illegible]ytop[illegible], N. [illegible]icala*, L. Moonen, L. den Hartigh, L. Mangiante, L. M. V. La[illegible], F. M. Dost, J. Beumer, S. Levy, R. S. van Leeuwaarde, W. [illegible]ong, K. Samsom, C. Voegele, A. Sexton-Oates, H. Begthel, J. [illegible]ving, L. M. Hillen, L. A. A. Brosens, S. Lantuejoul, S. Jaksani, M. F. M. Kok, K. J. Hartemink, H. M. Klomp, I. H. M. Borel Rinkes, A-M. C. Dingemans, G. D. Valk, M. R. Vriens, W. A. Buikhuisen, J. van den Berg, M. Tesselaar, J. L. Derks, E. J. M. Speel, M. Foll, L. Fernandez-Cuesta, H. Clevers

*Equally contributing first authors

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CHAPTER 10

General discussion

General discussion

Pulmonary carcinoid is an orphan disease accounting for 1-2% of all lung malignancies.[1,2] According to the World Health Organization (WHO) 2021 criteria, pulmonary carcinoids can be morphologically subdivided into low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) based on the mitotic rate (<2/mm^2 for TC and between 2 to 10 for AC) and the presence of necrosis (absent in TC and present in AC).[3] The ratio between TC and AC diagnosis is about 8-10:1.[4] While most pulmonary carcinoids can be curatively treated following surgical resection, distant relapse may arise up till 15 years after primary resection, occurring in 1-6% of patients with TC and 14-29% of patients with AC.[5-9] Known predictors for disease relapse, such as AC, lymphatic involvement, and tumor size, are unable to reliably exclude patients from long-term follow-up.[6,8,9]. Consequently, several guidelines advise a rather intensive follow-up of 10-15 years for all pulmonary carcinoid patients, while only a small proportion of all patients suffering from pulmonary carcinoid develop relapse of disease.[4,10,11] This thesis sheds light on the clinical dilemmas in clinical care of patients with pulmonary carcinoids using a Dutch nation-wide population-based cohort (2003-2012), investigates molecular mechanisms underlying tumorigenesis, identifies and verifies molecular biomarkers to improve prognostication, and describes the development of a new primary culture model for pulmonary carcinoid. All together the data in this thesis may lead to an improvement of current clinical management of patients suffering from pulmonary carcinoid.

Even though it is known that the currently applied criteria for pulmonary carcinoid diagnosis are subject to several limitations, they have remained largely unchanged since the introduction of the third edition of the WHO classification in 1999.[12] As a result, application of the WHO criteria may entail some important clinical dilemmas. One major limitation of the current WHO criteria is the poor reproducibility in distinguishing TC and AC on surgical resection specimen. The poor reproducibility is supported by several interobserver studies, in which agreement between pathologists is represented in kappa scores ranging from a moderate kappa of 0.60 to 0.76 (agreement required of 3 out of 5 pathologists, n=20 carcinoids) to a minimal agreement kappa of 0.32 (agreement required of 5 out of 5 pathologists, n=114 carcinoids).[13,14] Another study focusing on mitotic count regardless of necrosis revealed a median kappa of 0.21 for the delineation of TC from AC.[15] As the assessment of these morphological features is already challenging on correctly handled and optimally fixed surgical resection specimen, the WHO 2021 classification discourages a diagnosis of TC or AC on biopsy specimen.[3] This thesis provides the first population-based study to support this advice, as 57% (n=189/330) of pulmonary carcinoid patients exhibited discordance between the

preoperative biopsy (endo/transbronchial and transthoracic) and paired resection specimen diagnosis (**Chapter 3**). Moreover, 25% of patients preoperatively diagnosed as TC or carcinoid NOS were reclassified as AC after evaluation of the resection specimen. Other studies evaluating the accuracy of the WHO criteria on small biopsy specimen are scarce. Nevertheless, a study of Jamal *et al*. emphasized the difficulty of obtaining an accurate carcinoid diagnosis on preoperative biopsies.[16] Results showed that in only 47% of the 38 patients who underwent a bronchoscopic biopsy the correct carcinoid diagnosis was established.[16] Furthermore, a recent study suggested that the accuracy of a preoperative biopsy is dependent upon the cumulative biopsy size.[17] Data showed that an AC diagnosis was consistently missed in biopsies < 4mm^2 resulting in the advice to consider the biopsy surface in carcinoid classification. Nevertheless, the study is partly biased since the cohort, consisting of central/bronchial located tumors, is gathered in a referral centre for endobronchial treatment. Endobronchial treatment, with a rigid bronchoscope, results in large biopsies and in selected cases even in complete resection. Moreover, it needs to be emphasized that it may not always be possible to obtain biopsies of a size greater than 4 mm^2. The unreliable preoperative diagnosis encounters a serious clinical dilemma especially since a recent surgical trend is in favour of parenchyma sparing resections (e.g., segmentectomy) as compared to lobectomy or pneumonectomy as it may reduce morbidity.[12] Several recent retrospective studies have shown non-inferiority for parenchyma-sparing resections (e.g., wedge resection and segmentectomy) compared to the traditionally advised lobectomy for TC, while lobectomy remains the treatment of choice for AC.[18-27] A Japanese phase 3 trial has investigated whether segmentectomy was non-inferior to lobectomy in patients with small-sized peripheral non-small cell lung cancer (NSCLC).[28] Patients were enrolled to receive lobectomy (n=554) or segmentectomy (n=552). Results showed that the 5-year overall survival was 94.3% (92·1-96·0) for segmentectomy and 91.1% for lobectomy (95% CI 88·4-93·2) and relapse-free survival was 88.0% (95% CI 85·0-90·4) for segmentectomy and 87.9% (84·8-90·3) for lobectomy (HR 0·998; 95% CI 0·753-1·323; p=0·9889).[28] These data indicate that segmentectomy is non-inferior and superior to lobectomy for overall survival in patients with small-sized (≤2cm), thereby suggesting that segmentectomy should be the standard surgical procedure.[28] These data are further supported by a European multicenter non-inferiority phase 3 trial in which NSCLC patients clinically staged as T1aN0 ≤2 cm (diameter), were randomly assigned to lobar or sub-lobar resection.[29] Results again revealed that sub-lobar resection was noninferior to lobar resection regarding the 5-year disease free survival rates (e.g., 63.6% and 64.1%, respectively). Yet, surgical treatment decisions desire accuracy of the preoperative biopsy classification which is currently lacking for pulmonary carcinoids. Therefore, we advise clinicians to interpret the preoperative biopsy diagnosis with caution in deciding the extent of surgery.

Despite the relative indolent nature of carcinoid tumors, relapse rates after a surgical resection range between 1-6% for patients with TC and 14-29% for patients with AC. Current guidelines recommend routine surveillance, for an extensive period differing per guideline, with computer tomography (CT) scan of the thorax/abdomen including liver imaging, after surgical resection. Nevertheless, it needs to be considered that these recommendations are largely based on retrospective (multi)institutional studies with a relatively limited follow-up considering the prolonged time to relapse in pulmonary carcinoid patients. For example, Lou *et al.* performed an institutional study concerning 337 patients with a median follow-up time of 3.5 years and showed that 21 patients (6%) experienced relapse of disease[8]. Another study by Lee *et al.* revealed that 7 of 142 patients (6%) recurred following complete resection with a median follow-up of 2.6 years.[30] In **chapter 2**, we established a population-based cohort, mainly focusing on relapse of disease, by utilizing a unique combination of two Dutch registries with nation-wide coverage (Palga and the Netherlands Cancer Registry (NCR)). Evaluation of the cohort (N=662) revealed a median follow-up of 87.5 months and showed that approximately 10% of patients diagnosed with pulmonary carcinoid disease develop relapse after surgical resection. The median time to local disease relapse was 27.5 (95% CI 0-64.7) and to distant relapse 50.8 (95% CI 36.9-64.7) months. Distant relapses mostly occurred within 8 years and almost all within 10 years after resection. Therefore, our evaluation highlights the importance of long-term follow-up as studies providing short(er) follow-up can deliver false negative results on relapse of disease. This is especially important to consider when performing biomarker studies focussing on prediction of disease relapse.

Because a high proportion of patients with a pulmonary carcinoid will not develop relapse following complete resection, routine postoperative imaging surveillance may not be warranted for all patients. Hence, alterative follow-up strategies focusing on relapse risk are desired. Previous studies have identified that the highest relapse rates are observed in patients diagnosed with an AC which makes this diagnosis a strong clinical predictor for relapse.[12] **Chapter 6** evaluated the current WHO 2021 classification in relation to relapse of disease in a population-based cohort. All patients with surgically resected pulmonary carcinoid disease (2003-2012) were identified from the Dutch cancer/pathology registry and a matched relapse vs. non relapse cohort (ratio 1:2, N=161) was constituted. Cases were WHO revised in a blinded fashion by four pathologists. While most studies focus on the ability of a classification to select patients at risk for disease relapse (positive predictive value (PPV)), **chapter 6** focusses mainly on selecting patients at low risk for disease relapse (negative predictive value (NPV)) as these patients might benefit from shorter follow-up. In addition, management of risks using NPV is more reliable in a population with a low a-priori chance compared to PPV

which requires a high a-priori chance. Results revealed that pathologist seem to correctly predict relapse mainly in patients diagnosed as AC. Nonetheless the NPV of the WHO classification is still insufficient as 61.4% (n=35/57) of patients who experienced a relapse had a TC diagnosis. On top of that, WHO revision results of the matched cohort again revealed poor agreement among pathologists for the WHO criteria separating TC and AC (kappa mitotic count: 0.38, kappa necrosis: 0.48). Together, these data indicate the need for additional clinical predictors. Several studies have reported tumor size and lymphatic involvement as predictors of disease relapse.[3,6,8,9] However, despite yielding highly prognostic information and being recommended by European Association of Thoracic Surgeons (ESTS) guidelines, complete lymphadenectomy is rarely performed in routine care (7.0% (n=38/546)) (**chapter 2**).[31] Results in **chapter 2** show that lymph node sampling does not have a remarkable impact on disease relapse, whereas a study by Mineo *et al*. reports that systematic lymphadenectomy and immunohistochemical detection of micro metastases improved staging and identification of patients at risk for relapse.[32] In addition, lymph node metastases are not uncommon in pulmonary carcinoids and may be present in up to 21% of patients.[11,33] Furthermore, sampling provides important prognostic information, thus systemic nodal dissection is highly recommended.

Nowadays, biomarkers have become increasingly more relevant in predicting patient's prognosis and disease outcome. While several prognostic markers for pulmonary carcinoids have been identified in research settings (Ki-67, OTP, CD44, MEN1), they have not yet been implemented in routine clinical care.[3] The Ki-67 proliferative index (PI), for example, is included in the grading system of gastrointestinal neuroendocrine tumors.[34] Nevertheless, contradictory findings on the prognostic value and inconsistencies in proposed cut-off values has hampered implementation of the KI-67 PI as a diagnostic criterion for pulmonary carcinoids. By expression profiling, loss of *OTP,* alone or in combination with *CD44,* have been identified as molecular markers that enable the identification of patients at risk to develop disease relapse.[35,36] To date several studies have investigated the combination of these markers in the prognostication of pulmonary carcinoids.[37,38] A recent study of Centonze *et al*. examined the role of Ki-67 PI in disease evolution and its correlation with amongst others OTP and CD44 in a multicentre cohort of 317 patients. This cohort was subdivided into a low-Ki67 (<3%) and high-Ki67(≥3%) group following receiver operating characteristic (ROC) curve analysis to identify patients with early relapse (within 4 years from surgery).[38] OTP was significantly more often expressed in the low-Ki67 cohort, whereas no significant difference for CD44 was observed. The study mainly focusses on the role of Ki-67 alone to identify patients at risk for early relapse rather than a multimarker panel predicting relapse of disease. Reuling *et al*. showed in a single institutional cohort consisting of 171 patients with bronchial

carcinoid that a Ki-67 index of ≥5% was significantly associated with distant metastases as well as loss of OTP and CD44 protein expression.[37] Survival analysis showed a significant difference in distant metastasis-free survival in biomarker-based categories of patients. Patients with a favourable profile (mitotic count <2 per 2mm^2, Ki-67<5%, OTP≥30, CD44≥30) did not develop distant metastasis during follow-up. In contrary, we show, in **chapter 6**, that 13 patients (n=7 patients with endobronchial located tumor) harbouring these characteristics relapsed. However, it needs to be emphasized that the Reuling *et al*. cohort may be biased as the cohort is established within a national referral centre for the treatment of endobronchial tumors. Moreover, as mentioned before, these studies focus on selecting patients at risk for relapse while it may be more useful to select patients at low risk for disease relapse as these patients might benefit from shorter follow-up. **Chapter 6** focusses on the NPV rather than the PPV. IHC risk stratification (Ki-67, OTP, and CD44) of the total cohort, regardless of WHO classification, showed a significantly different relapse free survival between predicted high-risk (n=220) and low-risk (n=314) patients with a NPV of 95.9%. In other words, among those who had a low-risk IHC profile, the probability of being disease-free was 95.9%. Most importantly, reliability analysis for the IHC markers (Ki-67, OTP, and CD44), that were scored independently by 4 pathologists, revealed an excellent overall agreement for all markers (kappa Ki-67 score: 0.917, kappa OTP score: 0.984, kappa CD44 score: 0.976). Thus, application of the IHC marker panel, regardless of WHO classification, resulted in more accurate prediction of relapse post-surgery as well as more conformity among pathologists. Nevertheless, these findings should be validated in an independent pulmonary carcinoid patient cohort and prospective studies should explore the safety of a biomarker driven follow-up management. Moreover, future studies should investigate the applicability of this marker panel on preoperative biopsies to improve both the reliability of the preoperative diagnosis as well as the prognostication to select patients that might benefit from parenchymal sparing surgery. In addition, it would be of interest to evaluate expression of these markers in patients with metastatic pulmonary carcinoid to further investigate the PPV of the IHC marker panel.

Pulmonary carcinoids differ from high-grade neuroendocrine tumors as well as NSCLC based on their gene mutation spectrum.[39,40] Pulmonary carcinoids exhibit low mutational burden (0.4 mutations/Mb). The most frequently identified mutations occur within Multiple Endocrine Neoplasia 1 (*MEN1*) and are associated with a poorer prognosis.[40-42] Other frequently mutated genes (*ARID1A*, *EIF1AX*, members of the SWI/SNF complex) are implicated in the chromatin remodelling pathway, a process that controls gene expression.[40] However, none of them are currently used to subdivide patients into different carcinoid subtypes, and as a result subdivision remains to rely on morphology only. Recently, a multi-omics paper of Alcala *et al*. reported the existence of three well-

characterised molecular clusters of pulmonary carcinoids (carcinoid A1, A2, and B) with different prognoses and clinical implications.[43] Moreover, their study unravelled six samples within the carcinoid A1 cluster that shared molecular and clinical features with LCNEC while their morphology matched with carcinoids. These so-called ''supra-carcinoids'' may imply a possible molecular link between the low-grade and high-grade neuroendocrine neoplasms. Differential expression analysis between the three molecular clusters identified several core differentially expressed genes that were associated with survival, *OTP* being one of them.[43] The existence of these three clusters was confirmed by another multi-omics study.[44] This study of *Laddha et al.* identified two key biomarkers (*ASCL1* and *S100*) that together enable stratification of the three carcinoid clusters (named as LC1, LC2, and LC3). Both studies showed that one cluster (carcinoid A1 and LC1) was enriched for older female patients with peripheral tumors while another cluster (carcinoid A2 and LC3) was enriched for younger patients with mainly endobronchial situated tumors. Integrative analysis of both studies revealed that the three molecular subclusters could be separated, both on the mRNA and protein level, by application of three biomarkers (OTP, HNF1a, and ASCL1) (Moonen *et al.* manuscript in preparation, data not shown in this thesis). Results revealed strong prognostic relevance and unique clinical features, independent of typical/atypical histology, for the different clusters. Together, these data suggest that despite having low mutational burden, pulmonary carcinoids can be separated into unique carcinoid subtypes based on multi-omics analysis and provide deeper insight into their molecular mechanisms underlying tumorigenesis. Moreover, these different carcinoid subtypes may benefit from different therapeutic treatment modalities.

The absence of a reliable *in vitro* model of pulmonary carcinoids has hampered investigation of neuroendocrine tumor biology and drug development. Two human cell-lines are currently available (NCI-H727, NCI-H720) but it is debated whether these cell-lines truly mimic pulmonary carcinoid behaviour. **Chapter 9** describes the first patient-derived tumor organoids (PDTOs) from pulmonary neuroendocrine tumors. PDTOs are 3D cultures of tumor cells that can be expanded long-term as well as cryopreserved. To date, a small amount of PDTOs have been generated from high-grade neuroendocrine tumors including small cell lung cancer (SCLC), pulmonary large cell neuroendocrine carcinomas (LCNEC), and grade 3 gastroenteropancreatic neuroendocrine tumors and carcinomas.[45-48] Previous data proved that PDTOs are representative of the patient's tumor tissue from which they were derived at both the genetic and phenotypic level.[49-52] These findings are in line with our data showing that PDTOs preserve the histopathological profile, the gene expression patterns, the intratumor heterogeneity, and evolutionary processes of the parental tumors. Pulmonary carcinoid PDTOs, thus, provide a unique model to test, amongst others, therapeutic vulnerabilities. Currently,

treatment of choice for patients with pulmonary carcinoid disease is surgical resection. Nevertheless, the growth factor dependency analyses showed that pulmonary NET PDTOs are EGF-dependent and that these PDTOs express the EGF-receptor. This finding provides rational for future investigations to assess whether the EGF-receptor may be a possible treatment target for pulmonary carcinoids patients. In addition, **chapter 7** revealed that differential *OTP* mRNA expression is associated with differences in DNA methylation. Overall, OTP^{low} carcinoids exhibit higher DNA methylation levels as compared to OTP^{high} carcinoids thereby suggesting that in tumors of patients with unfavourable prognosis, OTP expression is lost, most likely due to high DNA methylation levels. These findings raise the question whether epigenetic therapies might be useful in the treatment of patients with pulmonary carcinoids. Epigenetic therapies seek to normalize DNA methylation patterns and post-translational modification of histones that promote or maintain a malignant phenotype.[53] Nine epigenetic agents are currently available for standard-of-care treatment in the United States, amongst others, two DNA methyltransferase (DNMT) inhibitors. DNMT inhibitors (i.e., azacytidine and decitabine) are antimetabolites that inhibit DNMT activity and induce hypomethylation when incorporated into DNA.[53] The efficacy of azacytidine in NETs has been tested *in vitro* using H727 (pulmonary), CNDT2.5 (midgut), and BON1 (gastrointestinal) cell lines. Azacytidine initiated a dose-dependent reduction of the proliferation rate of the cell lines and induced G2/M cell growth arrest.[54] Future studies may investigate whether gene specific epigenetic therapies may provide new potential therapeutics for pulmonary carcinoid patients.

In conclusion, this thesis reveals that disease relapse in pulmonary carcinoid patients is not rare (10%) and that, despite yielding highly prognostic information, lymph node involvement is rarely comprehensively assessed using complete lymphadenectomy in routine care (7%). Furthermore, it provides evidence that preoperative pulmonary carcinoid diagnosis on biopsy specimen is imprecise as 57% of the patients exhibits discordance between the biopsy and paired resection specimen diagnosis. Moreover, the poor reproducibility of the WHO classification criteria for pulmonary carcinoid, the inapplicability of these criteria on preoperative biopsy specimen and the modest prognostic significance reflects the need for a tailored classification system specific for this rare disease. An adjusted classification established on relapse free survival identifying patients with low risk of relapse that might benefit from parenchyma-sparing surgery and shorter follow-up should rather be the endpoint. The WHO criteria should serve as a basis for pathologist to identify pulmonary carcinoid disease and the addition of molecular markers should provide a framework to subclassify patients into prognostically relevant categories thereby refining clinical care. Future prospective studies should investigate the safety and applicability of a biomarker driven follow-up in

which patients will be allocated, based on the tumors immunohistochemical profile, to a low-risk of relapse and high-risk of relapse group. Subsequently, patients assigned to the low-risk group might benefit from shorter follow-up with less radiological exposure. In addition, studies should examine the ability of the biomarker panel on preoperative biopsies to identify patients who are at low risk for disease relapse. For these patients, and especially in patients with high cardiovascular comorbidity or limited lung capacity, clinicians may consider parenchyma-sparing surgery thereby preserving lung function. Most importantly, the alternative biomarker driven follow-up strategy should be discussed with the patient as part of shared decision making. Altogether, the results in this thesis may support the implementation of molecular markers for pulmonary carcinoid patients to refine clinical care.

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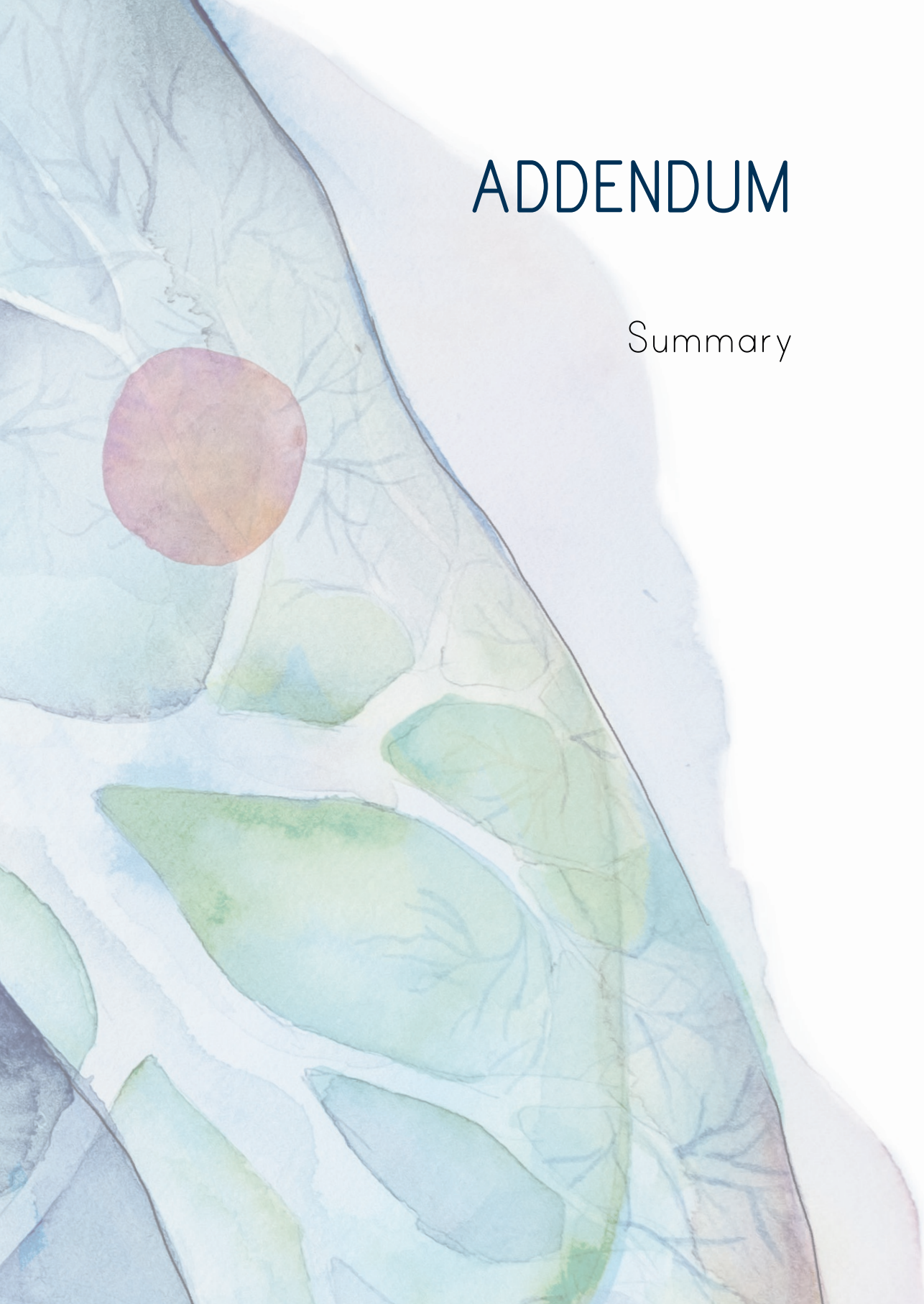

ADDENDUM

Summary

Summary

Pulmonary neuroendocrine neoplasms (NEN) encompass the well differentiated typical and atypical carcinoids (TC and AC) as well as the poorly differentiated neuroendocrine carcinomas large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) (**chapter 1**). Pulmonary carcinoids account for approximately 1-2% of all lung malignancies and their incidence is rising over the last decades. According to the World Health Organization (WHO) 2021 classification criteria, pulmonary carcinoids can be morphologically subdivided into TC and AC based on the mitotic count (TC <2/2mm^2, AC 2-10/2mm^2) and the presence or absence of necrosis. Although pulmonary carcinoids are considered as low- and intermediate grade tumors, distant disease relapse may occur (TC: 1%-6% and AC: 14%-29%) in patients who initially underwent curative surgical resection. Relapse may occur many years after surgical resection, hence the recommendation for long-term surveillance (10-15 years). Known predictors of distant relapse are AC, lymphatic involvement, and incomplete resection status. However, none of them can be reliably used, alone or in combination, to safely exclude patients from long-term follow-up. Moreover, the histopathological classification criteria for pulmonary carcinoids are subject to high-interobserver variation. Together these data indicate the need for new biomarkers to improve both the diagnosis and prediction of prognosis of pulmonary carcinoid patients. Currently, several prognostic markers (OTP, Ki67, CD44) for pulmonary carcinoids have been identified in research settings, but they have not yet been implemented in routine clinical care.

The **aim** of this thesis was 1) to examine the diagnostic workup and prognostication of pulmonary carcinoids in current clinical practice using a unique population-based cohort, 2) to obtain insights into molecular mechanisms underlying carcinoid tumorigenesis, 3) to identify and verify molecular biomarkers to improve the prognostication of pulmonary carcinoid patients, and 4) to develop the first patient-derived tumor organoids for pulmonary NENs to uncover therapeutic vulnerabilities.

Lymph node sampling in relation to disease relapse

The predictive value of extent of per-operative lymph node sampling in relation to disease relapse in pulmonary carcinoid patients is unknown. In addition, post-surgery follow-up recommendations rely on expert opinions and institutional retrospective studies with a rather short follow-up focusing mainly on overall survival instead of relapse free survival. In **chapter 2**, we aimed to address these shortcomings in a population-based cohort with long-term follow-up (median 87.5 months). By combining the Dutch pathology (Palga) and cancer registries (NCR), all patients with surgically resected pulmonary carcinoid disease diagnosed between 2003-2012 were included.

Tumor node metastasis (TNM) staging was updated to TNM8 by screening of complete pathology reports. Both patterns of metastasis and extent of lymph node sampling were evaluated. In total 662 patients were included in the study of which 10% showed disease relapse (26.0% AC vs. 6.2% TC vs. 4.4% carcinoid not otherwise specified (NOS)). Relapses occurred mostly in the liver (50%) and locoregional sites (45%). Median relapse free interval (RFI) was 48.1 months (95% CI 36.8-59.4) thereby underscoring the necessity of long-term follow-up. Poor prognostic factors were AC, pathological nodal stage (pN1/2) and surgical resection margin (R1/R2). Data regarding lymph node dissection were available in 546 patients. A complete mediastinal lymph node sampling according to the European Society of Thoracic Surgeons (ESTS) guidelines was performed in merely 7% of the patients. A slight increase in mediastinal lymph node evaluation was observed over time but this was not associated with the number of relapses. In 477 clinical N0 patients, 5.9% showed pathological N1 disease and 2.5% N2 disease. These data indicate that the extent of lymph node sampling has not a remarkable impact on disease relapse, however, systematic nodal evaluation is advocated as it provides important prognostic information. Furthermore, relapse of disease is not uncommon, and our data show that a long-time follow-up is required whereby surveillance should especially focus on liver and locoregional relapses as these were the most frequent sites of relapse during follow-up.

Preoperative biopsy specimen diagnosis in pulmonary carcinoid

In **chapter 3**, we evaluated the Dutch pathology database to select stadium I-III pulmonary carcinoid patients who underwent a curative resection and of whom both a preoperative biopsy and paired resection specimen were available (n=330). Pathology report conclusions of the biopsy and paired resection were compared. This evaluation showed discordance between the preoperative biopsy and paired resection diagnosis in 57% (n=189/330) of the patients. Moreover, a quarter of preoperatively diagnosed TC and carcinoid NOS patients were reclassified as AC on the resection specimen and these patients exhibited higher relapse rates as compared to non-reclassified TC and carcinoid NOS patients (3% vs. 1% and 16 vs. 6%). Therefore, we advise clinicians to interpret the preoperative biopsy diagnosis with caution in deciding the extent of surgery (e.g., parenchyma-sparing versus non-parenchyma sparing). In **chapter 4**, we emphasize the need for additional preoperative biomarkers that aid in both diagnosis and prognosis of pulmonary carcinoids. We feel that a panel of molecular markers (e.g., OTP, Ki67, CD44) applicable on preoperative biopsies may improve the diagnostic and prognostic accuracy to predict relapse in patients suffering from pulmonary carcinoids disease.

Orthopedia homeobox (OTP) in pulmonary carcinoids

OTP is a member of the homeodomain transcription factor family and has been described as a key player in the development of the neuroendocrine system of the hypothalamus. The current clinical value of OTP expression identified in pulmonary carcinoids, the possible molecular mechanism regulating OTP expression, and the function of OTP are addressed in a literature review in **chapter 5**. This evaluation underscores that OTP is a promising, highly sensitive, and specific marker for pulmonary carcinoid tumors with a favourable prognosis. However, at time of evaluation only a limited number of tumor types had been examined for OTP expression and the regulatory mechanism underlying OTP expression remained undetermined. Hence, in **chapter 7**, we investigated publicly available multi-omics data (whole-exome-, whole-genome-, RNA sequencing and Epic 850K-methylation array) of 58 TC, 27 AC, 69 LCNEC and 51 SCLC patients and TCGA (The Cancer Genome Atlas) data of 33 tumor types 1) to shed light on *OTP* expression patterns in different tumor types and 2) to unravel the mechanisms underlying differential *OTP* expression. Results showed bimodality of *OTP* expression in carcinoids (OTP^{high} vs. OTP^{low} group), with the OTP^{high} group specific to pulmonary carcinoids while absent from all other cohorts analysed. OTP^{low} carcinoids showed a statistically significant worse overall survival. Gene set enrichment analysis (GSEA) for mutated genes related to hallmarks of cancer revealed robust enrichment of three hallmarks in the OTP^{low} group (sustaining proliferative signalling, evading growth suppressor and genome instability and mutation) whereas no robust enrichment was observed within the OTP^{high} group. To date, no gene-inactivating somatic mutations, alterations by chimeric transcripts or genomic rearrangements have been identified in the *OTP* gene. Therefore, we examined epigenetics (e.g., DNA methylation) as a potential regulatory mechanism underlying differential OTP expression by combining transcriptomic and methylomic data of 51 samples (24 OTP^{high}, 10 OTP^{low}, and 17 LCNEC samples). Analyses identified a significantly different methylation level (FDR <0.05 and delta >0.2) between OTP^{high} and OTP^{low} carcinoids for 12/34 OTP 850K Infinium probes. Overall, OTP^{low} carcinoids exhibit higher DNA methylation levels as compared to OTP^{high} carcinoids. Together these data suggest that high OTP expression is a unique feature of pulmonary carcinoids with a favourable prognosis, and that in poor prognostic patients OTP expression is lost, most likely due to changes in DNA methylation levels. Future studies should investigate whether epigenetic therapies might play a role in the treatment of pulmonary carcinoid patients.

Prognostic markers for pulmonary carcinoids

Relapse occurs in 10% of patients with resected pulmonary carcinoid. While TC show relative low relapse rates, safe exclusion from long-term follow-up is impossible as these

patients may also develop disease relapses over time (1-6%). It might be that these patients are misdiagnosed due to the existing interobserver variation in the WHO classification suggesting that these patients were most likely AC but initially not recognized as such. **Chapter 6** examines if an immunohistochemical (IHC) marker panel (OTP, Ki67, CD44) improves 1) uniformity among pathologists (compared to morphological diagnosis) and 2) prediction of relapse free survival. For this purpose, all surgically resected pulmonary carcinoid (2003-2012) were identified from the Dutch pathology and cancer registry. Subsequently, a case-control cohort (2:1 for relapse, n=170) was established in which patients were matched on pathological T-status (pT), pathological N-status (pN), surgical resection margin (R0, R1, and R2), type of resection and application of adjuvant chemotherapy. The case-control cohort was revised by four pathologists using WHO criteria and IHC markers were assessed for H-score (OTP, CD44) and eyeball estimation (Ki67). The remaining total cohort was revised and scored in a similar way by two out of four pathologists (N=396/566). IHC cut-off values were determined using time dependent ROC curve analysis for relapse (OTP (<50), CD44 (<30), and Ki67 (≥5)). Agreement between pathologists for diagnostic classification and IHC was determined using kappa (k). Median follow-up of the case-control cohort was 86.7 months and 61% (n=35/57) of the relapsed patients had a TC diagnosis. WHO revision showed poor agreement among pathologists (mitotic count k: 0.38, necrosis k: 0.48), whereas assessment of IHC displayed high uniformity (Ki67: 0.92, OTP: 0.98, CD44: 0.98). Mean negative predictive value (NPV) for relapse increased from 0.74 (TC WHO diagnosis) to 0.85 (IHC low risk (OTP ≥50 & CD44 ≥30 & Ki67 <5)). IHC risk stratification of total cohort (high risk (n=220) and low risk (n=314)) showed a NPV of 96%. These results indicate that a biomarker driven follow-up management for pulmonary carcinoid patients may be used in the future to identify patients who can be excluded from long-term follow-up.

Discontinuation of the initially used OTP polyclonal antibody (pAb) and the absence of a reliable monoclonal antibody (mAb), however, hamper implementation of OTP into routine diagnostics. **Chapter 8** describes the development and verification of two new OTP mAbs (CL11222 and CL11225), produced from mice immunized with a recombinant human OTP protein fragment. Epitope-mapping and isotyping revealed that both mAbs were of IgG1 isotype but displayed different binding sites. The epitope of CL11222 corresponded to one of the epitopes of the pAb, while CL11225 recognized a unique epitope. Purified mAbs were validated for IHC in formalin fixed paraffin embedded (FFPE) normal peripheral tissues. Results showed the expected nuclear immuno-reactivity in a subset of neurons in human hypothalamus while no positivity was observed in any of the other tissues tested. Staining protocols were optimized on two automated staining platforms, i.e., Autostainer 480S (ThermoFisher) and Dako Link 48 (Agilent Technologies)

and results were cross validated with the OTP polyclonal antibody (pAb) (HPA039365, Atlas Antibodies). Immunostaining results on a pulmonary NEN tissue micro array showed positivity in 73.7% (n=28/38) TC, 64.3% (n=9/14) AC, 89.5% (17/19) carcinoid NOS, while SCLC and LCNEC (except for one case) remained negative. To verify the specificity of the mAbs for carcinoids of pulmonary origin, n=40 additional NENs of non-pulmonary origin were stained. All cases stained negative for both mAbs. Cross-platform validation results showed adequate OTP immunostaining and high concordance with the initially used rabbit pAb (kappa: 0.900). Inter-platform analyses revealed that mAb CL11222 displayed in five cases false negative results on the DAKO autostainers platform resulting in a preference for mAb CL11225. The excellent performance of the antibodies on FFPE material, the adequate concordance between different staining platforms and the good reproducibility allow application in routine diagnostics and may improve the current classification as well as the prognostication of pulmonary carcinoid patients.

Ex-vivo pulmonary neuroendocrine tumor (NET) model

Recently, several retrospective genomic studies have provided new insights into NEN subtypes and unravelled potential therapeutic targets. A reliable preclinical model that resembles the behaviour of these different NEN subtypes, however, is still lacking. In **chapter 9**, a collection of patient-derived tumor organoids (PDTOs) from multiple body sites were established, including pulmonary and extrapulmonary LCNEC as well as the first reported PDTOs from patients with pulmonary carcinoid. In total 20 NET (including 10 primary lung NETs) and 5 LCNEC PDTO lines were generated. Direct comparison of PDTOs and their matching tumor of origin revealed that PDTOs preserve the histopathological profile, the gene expression patterns, the intratumor heterogeneity, and evolutionary processes of the parental tumors. Drug sensitivity analyses revealed new potential sensitivities for LCNEC. In addition, growth factor dependency analyses showed that pulmonary NET PDTOs are EGF dependent and that these PDTOs express the EGF-receptor. Together these data provide a rational for future investigations to assess whether these targets may postulate targeted therapies on the horizon.

Discussion

Finally, in **chapter 10**, the results obtained in this thesis are discussed and reflected in light of the current available literature in the field. New insights acquired in this thesis may improve current clinical management of patients suffering from pulmonary carcinoid disease and provide valuable perspectives for future research.

ADDENDUM

Impact

Impact

In this thesis, 1) the reliability of the histopathological diagnosis and prognosis of patients suffering from pulmonary carcinoid is examined using a unique population-based cohort, 2) the molecular mechanisms underlying carcinoid tumorigenesis are investigated, 3) molecular biomarkers to improve the prognostication of pulmonary carcinoid patients are identified and verified, and 4) the first patient-derived tumor organoids for pulmonary neuroendocrine neoplasms to uncover therapeutic vulnerabilities are reported. In this impact chapter, we will place our findings into both a scientific and social perspective.

Pulmonary carcinoid is an orphan disease with an incidence ranging from 0.2/2 per 100,000 persons per year in both the United States and Europe.[1,2] Nevertheless, its occurrence has increased rapidly over the past decades mostly due to improved diagnostic techniques and increased awareness amongst clinicians. Moreover, pulmonary carcinoids are more often found by incident because people undergo scans for all kinds of diseases and conditions nowadays. Pulmonary carcinoids occur predominately at the fifth or sixth decade of life and can be morphologically (e.g., mitotic index and presence of necrosis) subdivided into low-grade typical carcinoids and intermediate grade atypical carcinoids.[3] The ratio between typical and atypical carcinoids is about 8-10:1. The post-operative median 10-year overall survival (OS) in patients with typical carcinoids is about 89% (range 83-100) and the disease-free survival (DFS) is 90% (range 73-95) [4]. In patients with atypical carcinoids the 10-year OS is 51% (range 38-74) and the DFS is 45% (range 24-71).[4] While most pulmonary carcinoids can be curatively treated by means of surgical resection, distant disease relapse occurs in 1-6% of patients with typical carcinoids and 14-29% of patients with atypical carcinoids.[5-9] Known predictors of distant relapse are atypical carcinoid, lymphatic involvement, and incomplete resection status. However, none of them can be reliably used, alone or in combination, to exclude patients from long-term follow-up. As a result, all patients require a rather intensive follow-up of 10-15 years dependent on the guideline used, while only a proportion of patients are at high-risk for relapse.[10-12] The extensive follow-up may cause morbidity, high anxiety levels and radiation exposure (due to (yearly) CT-scans) to patients, as well as substantial economic burden. The rising incidence together with the aging population, and the absence of reliable prognostic markers to safely exclude patients from long-term follow-up, could result in a continued increase of both disease and economic burden.

The requirement of additional reliable prognostic markers is illustrated by several treatment dilemmas within the management of pulmonary carcinoids. Findings in this

thesis show that the preoperative biopsy specimen diagnosis is unreliable in daily clinical practice resulting in discordance between the preoperative biopsy and paired resection diagnosis in 57% of patients (**Chapter 3**). The World Health Organization (WHO) criteria therefore state that definitive diagnosis is only feasible postoperatively.[3] However, in current clinical practice, treatment decisions are based on the pathological diagnosis of the preoperative biopsy. The preferred treatment for carcinoid disease is surgical resection, but in case of typical carcinoid parenchymal sparing strategies may be considered. Consequently, an imprecise preoperative diagnosis may result in over/under treatment regarding extent of surgery. In addition to imprecise preoperative biopsy diagnosis, our research group has previously shown that the WHO classification criteria (e.g., mitotic index and presence of necrosis) are subject to high-interobserver variation on postoperative resection specimen, affecting particularly atypical carcinoids.[13] This results in over-, and underestimation of the relapse risk as atypical carcinoid is a poor prognostic factor. Another poor prognostic factor for relapse risk is lymph node involvement as also reported in this thesis (**Chapter 2**). For this purpose, the European Society of Thoracic Surgeons guideline for intraoperative lymph node staging in non-small cell lung cancer recommends complete lymphadenectomy, but this is rarely (7%) performed in routine care of pulmonary carcinoid patients. Together these clinical dilemmas argue for the identification and implementation of additional markers to improve both diagnostic and prognostic carcinoid classification. In addition, clinicians are advised to interpret the preoperative biopsy diagnosis with caution in deciding the extent of surgery, and to always include a dedicated lymph node dissection as it provides prognostic information on disease relapse.

Despite the prognostic value of and research performed on molecular markers in pulmonary carcinoids, to date, none have been incorporated as a criterion in the WHO 2021 classification. Assessment of immunohistochemical expression of the nuclear protein MIB-1 (Ki-67) may improve current histopathological subclassification of pulmonary carcinoids (**Chapter 4**). Nevertheless, current studies evaluating the prognostic value of Ki-67 are contradictory and the absence of clear cut-off values separating typical from atypical carcinoids hampers diagnostic implementation into the WHO 2021 criteria. By expression profiling, we previously identified highly sensitive molecular markers to identify patients at risk for disease progression, i.e., orthopedia homeobox (*OTP*) alone or in combination with *CD44.*[14] The prognostic value of *OTP* has since been evaluated in larger series, confirming that loss of expression is associated with a poor prognosis (**Chapter 5**). Since these studies all contained selected institutional patient cohorts with incomplete follow-up, we validated the prognostic significance of *OTP*, *CD44,* and *Ki-67* in a large, unselected population-based cohort (**Chapter 6**). Results showed that the negative predictive value (NPV) of the marker panel was 95.9%,

indicating that our IHC marker panel can, regardless of WHO classification, reliably predict which patients will most likely not relapse over time. As a result, patients characterised with a low-risk IHC profile on the resection specimen may be excluded from long-term follow-up, which will benefit both patient and economic burden. When this high NPV withstands future prospective studies, the predictive marker panel may empower a biomarker driven post-surgery follow-up management.

To contribute to the implementation of reliable OTP immunostaining in routine diagnostics, new monoclonal OTP specific antibodies have been developed and verified, on two automated staining platforms (**Chapter 8**). Cross-platform assessment showed excellent agreement and good reproducibility on FFPE material. In addition, intratumor heterogeneity analysis showed that OTP is homogeneously expressed throughout the tumor resulting in a homogeneous staining pattern, indicating the applicability of OTP immunohistochemical staining on biopsy specimen. All together, these findings further encourage the implementation of OTP in routine diagnostics in both a pre- and postoperative setting to assist the pathologist in diagnostic decision making.

Recently, multi-omics analysis revealed three prognostically relevant molecular clusters of pulmonary carcinoids.[15] The existence of the three molecular clusters with distinct clinical features was further confirmed by a study of Laddha *et al.*.[16] Integrative genomic analysis of both studies revealed that expression of *OTP*, *HNF1a*, and *ASCL1* messenger RNA (mRNA) expression enabled sufficient separation of the molecular clusters. Subsequently, it could be shown that clustering based on mRNA expression strongly correlated with clustering based on protein expression of the three markers. Results revealed strong prognostic relevance and unique clinical features, independent of typical/atypical histology, for the different clusters (**data not shown in this thesis**).

The development of pulmonary neuroendocrine patient-derived tumor organoids (PDTO) facilitates studies investigating the molecular biology of neuroendocrine neoplasms to gather insights into the tumorigenesis. In addition, the model enables drug testing which may unravel therapeutic sensitivities for pulmonary neuroendocrine tumors (**Chapter 9**). Furthermore, we showed that differential *OTP* expression is associated with changes in DNA methylation levels (**Chapter 7**). Together these findings raise the question whether new potential therapies might be unravelled for pulmonary carcinoid patients and whether epigenetic therapies might play a role in the future.

The results and perspectives of this thesis contribute to the refinement of current clinical care by analyzing prognostic and diagnostic molecular markers, identify a prognostic marker panel that may allow a biomarker driven follow-up management for pulmonary

carcinoid patients in the future, provide insights in the management of follow-up after treatment, and reports a new primary culture model to derive insights into the biology of pulmonary carcinoids as well as possible new therapeutic targets. This has both scientific and economic impact, as it encourages the implementation of clinically relevant molecular biomarkers in current pulmonary carcinoid patient care. The clinical applicability of these molecular markers will eventually result in prognostically relevant patient subgroups. Hence, patients who have a low risk for relapse may benefit from shorter follow-up thereby reducing both health- and economic burden while high-risk patients are aided by a more dedicated follow-up.

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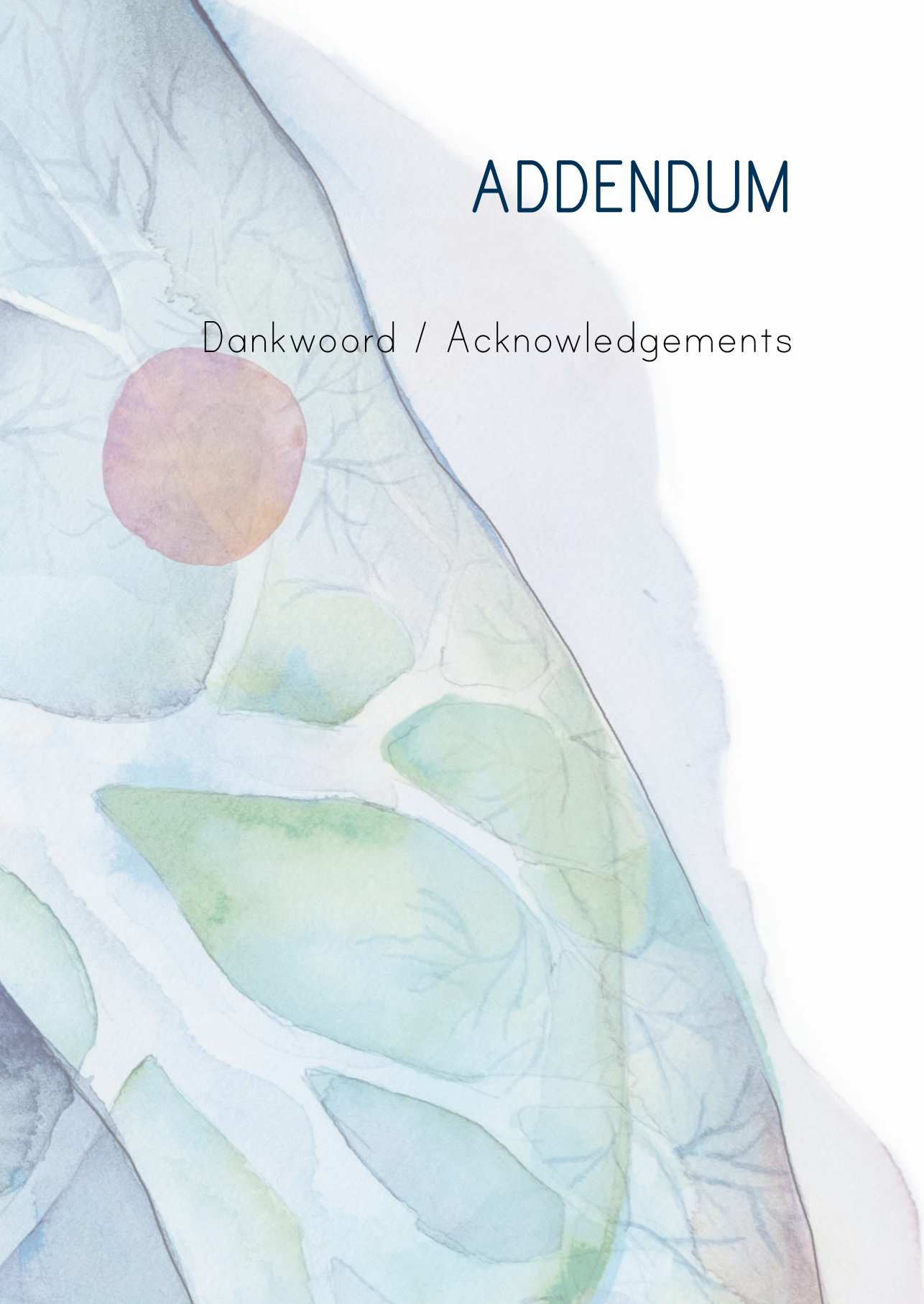

ADDENDUM

Dankwoord / Acknowledgements

Dankwoord

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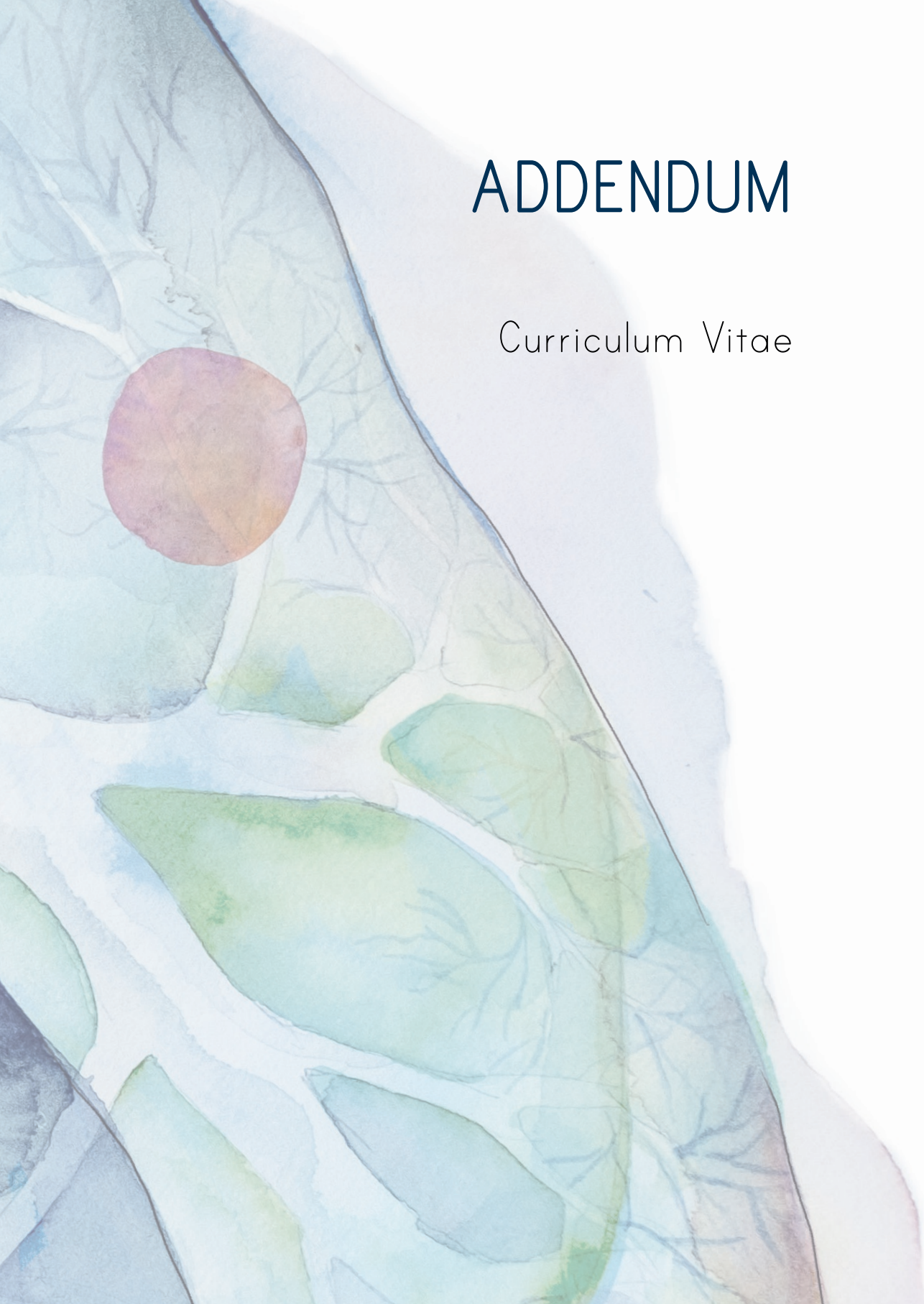


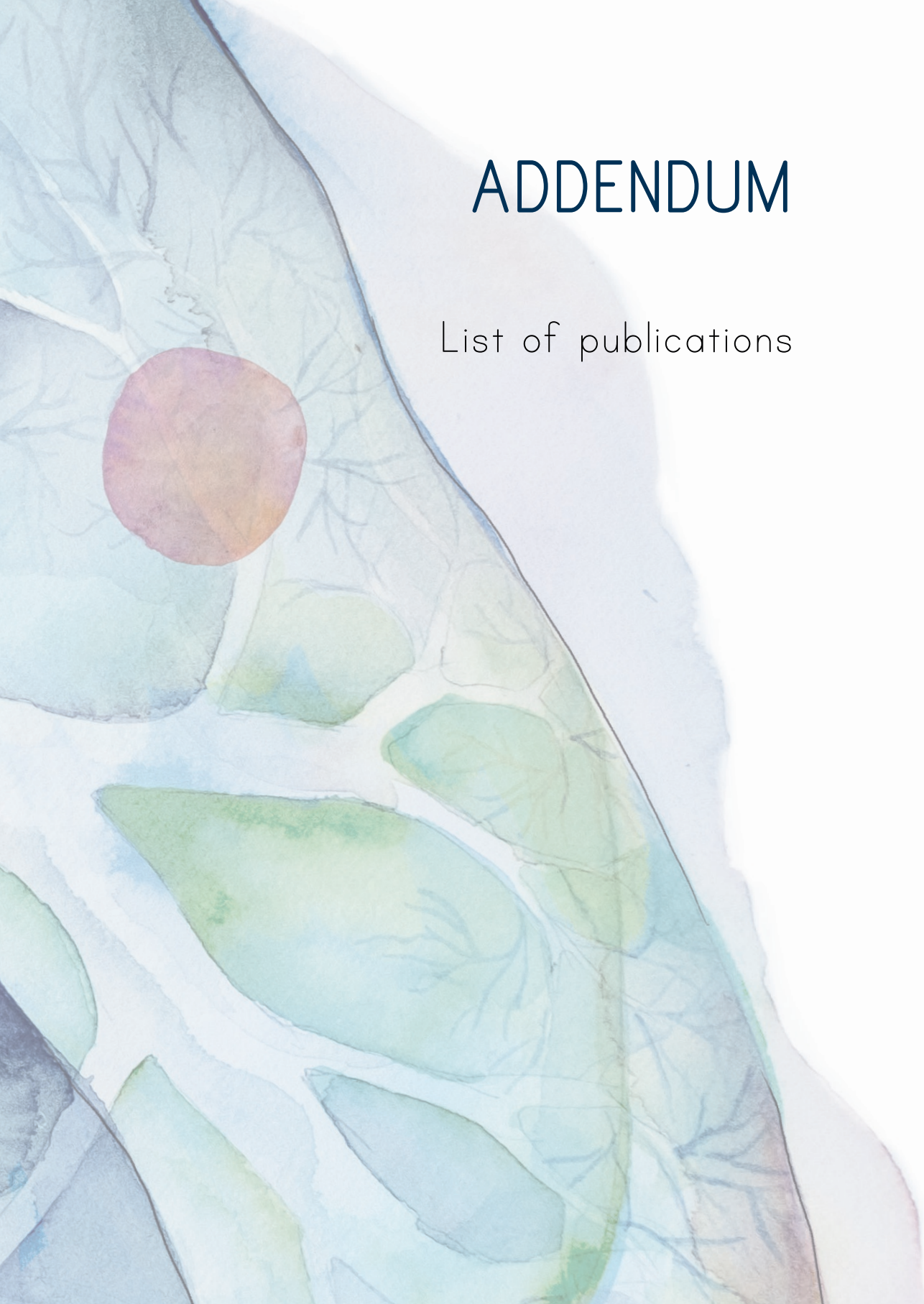
ADDENDUM

Curriculum Vitae

Curriculum Vitae

Laura Moonen was born on March 17th, 1995, in Weert, The Netherlands, as daughter to Theo and Marga Moonen and as a sister to Evy Moonen. In 2013 she graduated secondary school at Philips van Horne SG (Weert, The Netherlands). She decided to pursue her interest in biology and successfully completed her bachelor's degree in Health Sciences at Maastricht University in 2016. In 2018, she obtained her master's degree in Oncology and Developmental Biology, with distinction cum laude, at Maastricht University. In 2018, she started as a PhD candidate at the Department of Pathology at Maastricht University Medical Center (MUMC+) under supervision of Prof. Dr. Speel, Prof. Dr. Dingemans, and Dr. Derks. Her PhD project was focused on pulmonary carcinoids. During her PhD, she worked for two months as an intern at the International Agency for Research on Cancer (IARC, Lyon, France) to perform genomic analyses in pulmonary neuroendocrine tumors under supervision of Dr. Fernandez-Cuesta. Her work has been honored with two European Neuroendocrine Tumor Society travel grants and a Netherlands Respiratory Society travel grant. In addition, she received a financial grant of the Wassink Hesp Stichting to continue her work in the pulmonary neuroendocrine tumor field.

Currently, Laura is aiming to become a clinical molecular biologist in pathology at MUMC+ and Jeroen Bosch Hospital ('s-Hertogenbosch, The Netherlands) as trainee of Prof. Dr. Speel and Dr. van den Brule. It is expected that she will complete her training in December 2024. Additionally, she will keep advancing her scientific career, as a post-doctoral researcher in the neuroendocrine tumor field.



ADDENDUM

List of publications

List of publications

Published original research articles and reviews

Alcala, N., Leblay, N., Gabriel, A. A. G., Mangiante, L., Hervás, D., Giffon, T., Sertier, A. S., Ferrari, A., Derks, J., Ghantous, A., Delhomme, T. M., Charbrier, A., Cuenin, C., Abedi-Ardekani, B., Boland, A., Olaso, R., Meyer, V., Altmuller, J., Le Calvez-Kelm, F., Durand, G., Voegele, C., Boyault, S., **Moonen, L.**, Lemaitre, N., Lorimier, P., Toffart, A. C., Soltermann, A., Clement, J. H., Saenger, J., Field, J. K., Brevet, M., Blanc-Fournier, C., Galateau-Salle, F., Le Stang, N., Russell, P. A., Wright, G., Sozzi, G., Pastorino, U., Lacomme, S., Vignaud, J. M., Hofman, V., Hofman, P., Brustugun, O. T., Lund-Iversen, M., Thomas de Montpreville, V., Muscarella, L. A., Graziano, P., Popper, H., Stojsic, J., Deleuze, J. F., Herceg, Z., Viari, A., Nuernberg, P., Pelosi, G., Dingemans, A. M. C., Milione, M., Roz, L., Brcic, L., Volante, M., Papotti, M. G., Caux, C., Sandoval, J., Hernandez-Vargas, H., Brambilla, E., Speel, E. J. M., Girard, N., Lantuejoul, S., McKay, J. D., Foll, M., & Fernandez-Cuesta, L. (2019). Integrative and comparative genomic analyses identify clinically relevant pulmonary carcinoid groups and unveil the supra-carcinoids. Nature communications, 10(1), 1-21.

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