Original Article

Development and verification of new monoclonal orthopedia homeobox (OTP) specific antibodies for pulmonary carcinoid diagnostics

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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 (NETs) 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 microarray (TMA).

Results: Two clones (CL11222 and CL11225) were selected for purified monoclonal antibody (mAb) 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

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excellent agreement with the often-used polyclonal antibody allowing application in routine diagnostics.

Keywords: Pulmonary carcinoids; neuroendocrine; orthopedia homeobox (OTP); monoclonal antibody (mAb); prognostic

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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 (NETs) 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 NET tissue microarray (TMA). We present the following article in accordance with the STARD 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, 5.6 µm, 1 mL 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 1× phosphate buffered saline (PBS) was prepared. Antibody (2 µL) was mixed with 50 µL of master mix and 17 µL 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 ℃ 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 1:200–1:1,000 (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 (n=38), atypical carcinoids (n=14), carcinoid not otherwise specified (NOS, n=19), large cell neuroendocrine carcinomas (LCNEC, n=12), and small cell lung cancer (SCLC, n=3). All patients were diagnosed following the WHO criteria for pulmonary NETs (2).

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study

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

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

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).

TMA 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 (LH). 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.

IHC

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 1:3,000 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 20× 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 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.

Pathological assessment of OTP staining

Slides were assessed by an experienced pulmonary NET

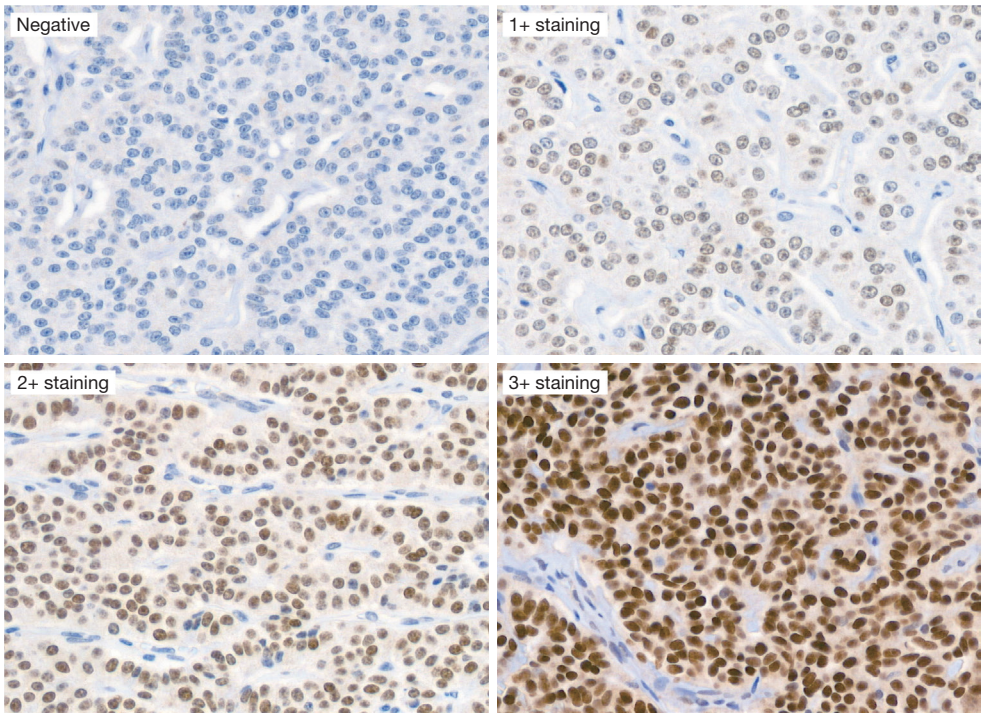

Figure 1 Representative immunohistochemical images showing the different OTP staining intensities (0, 1+, 2+, 3+) (magnification 20×). OTP, orthopedia homeobox.

pathologist and a researcher (LH, LM) 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 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 (Figure S1).

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

mAb 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 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 2A*).

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

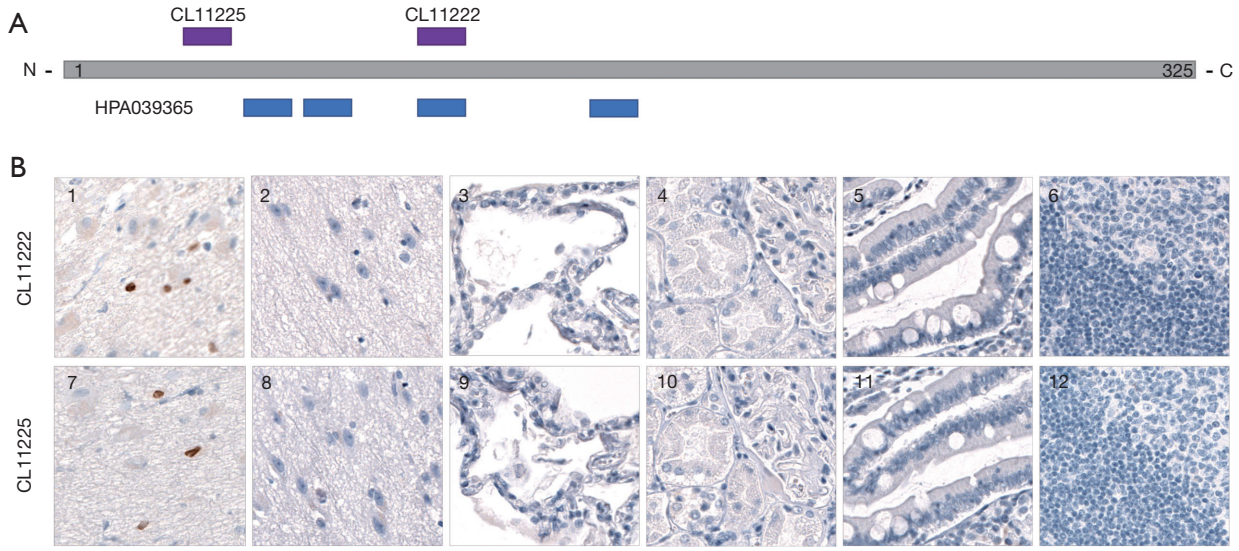

Figure 2 Overview of the mAb isotyping and characterisation. (A) Linear representation of OTP protein (in grey) showing binding sites of mAb CL11222 and CL11225 (in purple), as well as pAb HPA039365 (in blue). (B) Representative images illustrating OTP IHC of both CL11222 and CL11225 in [1, 7] human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

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 2B*). Titration experiments on the Thermofisher 480S autostainer revealed an optimal staining intensity at a 1:200 dilution.

mAb optimization for pulmonary neuroendocrine neoplasia

Immunostaining was further optimized on a PNEN TMA containing different histological subtypes [i.e., typical carcinoid (TC), atypical carcinoid (AC), carcinoid NOS, large cell neuroendocrine carcinoma (LCNEC), small cell lung cancer (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 3*).

Table 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 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 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 NETs, insulinomas, head and neck NETs, breast NETs, paragangliomas, and merkel cell carcinomas]. All cases stained negative for both mAbs.

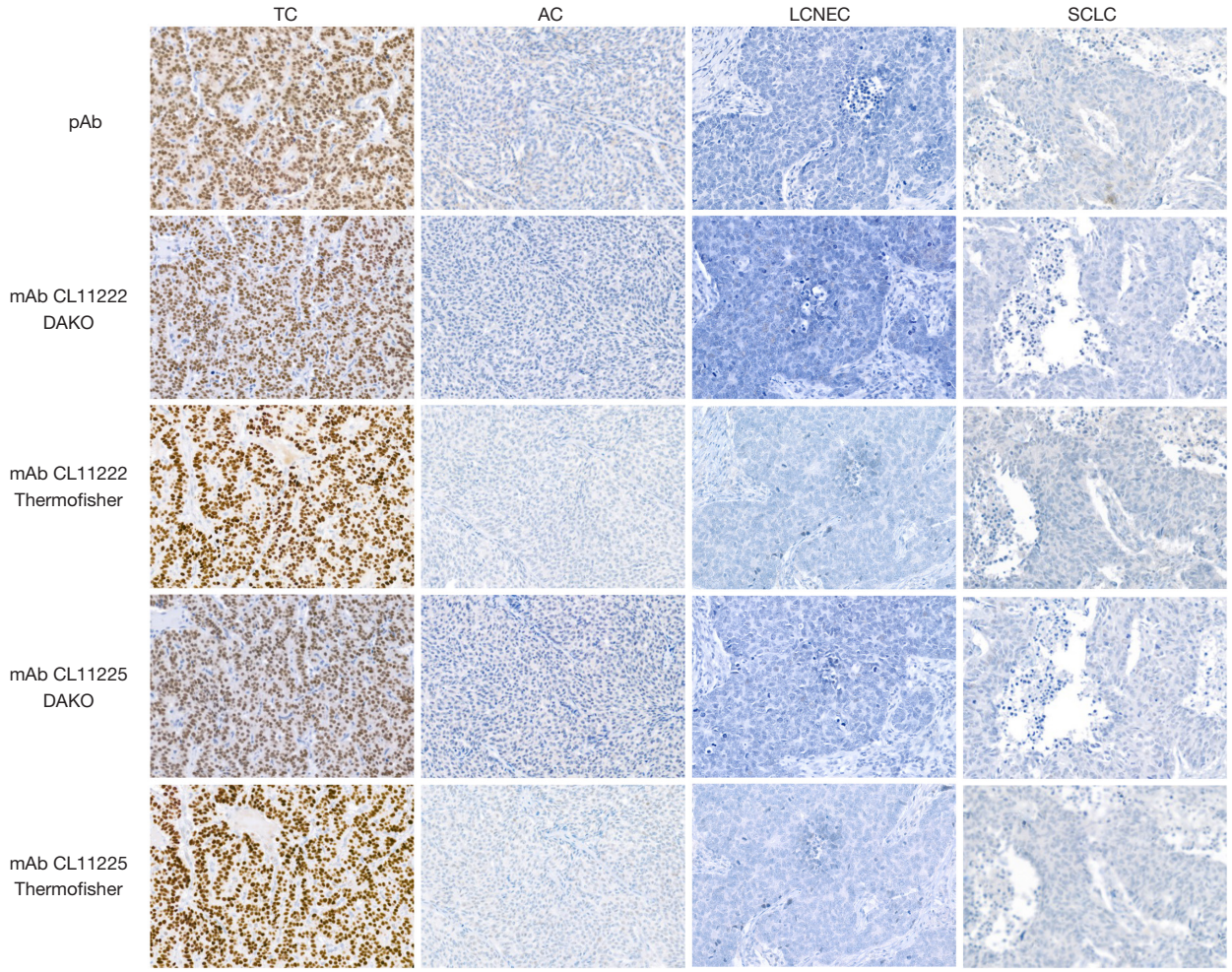

Figure 3 Representative images illustrating OTP immunohistochemical staining of pAb, CL11222 and CL11225 mAb in TC, AC, LCNEC, and SCLC (magnification 200×). pAb, polyclonal antibody; mAb, monoclonal antibody; TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; OTP, orthopedia homeobox.

Table 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

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.

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 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 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

ICC, intraclass correlation coefficient; CI, confidence interval.

Table 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

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 4*).

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 5*).

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

Table 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	–

pAb, polyclonal antibody; mAb, monoclonal antibody.

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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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-418/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-418/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-418/coif). EK is a stockholder of Atlas Antibodies AB. WT receives consulting

fees (to institution) from Merck Sharp Dohme and Bristol-Myers-Squibb. WT is a board member of the Dutch Society of Pathology, and a member of the council for Research and Innovation of the Federation of Medical Specialists. LMH received travel support of the European Thoracic Oncology platform to attend the ETOP 2022 meeting. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. 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).

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