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RESEARCH PAPER

The search for the primary tumor in metastasized gastroenteropancreatic neuroendocrine neoplasm

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Abstract Gastroenteropancreatic neuroendocrine tumors (NETs) often present as liver metastasis from a carcinoma of unknown primary. We recently showed that primary NETs from the pancreas, small intestine and stomach as well as their respective liver metastases differ from each other by the expression profile of the three genes *CD302, PPWD1* and *ABHB14B*. The gene and protein expression of *CD302, PPWD1,* and *ABHB14B* was studied in abdominal NET metastases to identify the site of the respective primary tumors. Cryopreserved tissue from NET metastases collected in different institutions (group A: 29, group B: 50, group C: 132 specimens) were examined by comparative genomic hybridization (Agilent 105 K), gene expression analysis (Agilent 44 K) (groups A and B) and immunohistochemistry (group C). The data were blindly evaluated, i.e. without knowing the site of the primary. Gene expression analysis correctly revealed the primary in the ileum in 94 % of the cases of group A and in 58 % of group B. A pancreatic primary was predicted in 83 % (group A) and 20 % (group B), respectively. The combined sensitivity of group A and B was 75 % for ileal NETs and 38 % for pancreatic NETs. Immunohistochemical analysis of group C revealed an overall sensitivity of 80 %. Gene and protein expression analysis of *CD302* and *PPWD1* in NET metastases correctly identifies the primary in the pancreas or the ileum in 80 % of the cases, provided that the tissue is well preserved. Immunohistochemical profiling revealed *CD302* as the best marker for ileal and *PPWD1* for pancreatic detection.

Keywords Neuroendocrine tumors · Carcinoma of unknown primary · Microarray · Genetic profile

Introduction

A carcinoma of unknown primary (CUP) syndrome is a common and challenging clinical problem. Despite intensive and costly diagnostics the primary can only be detected

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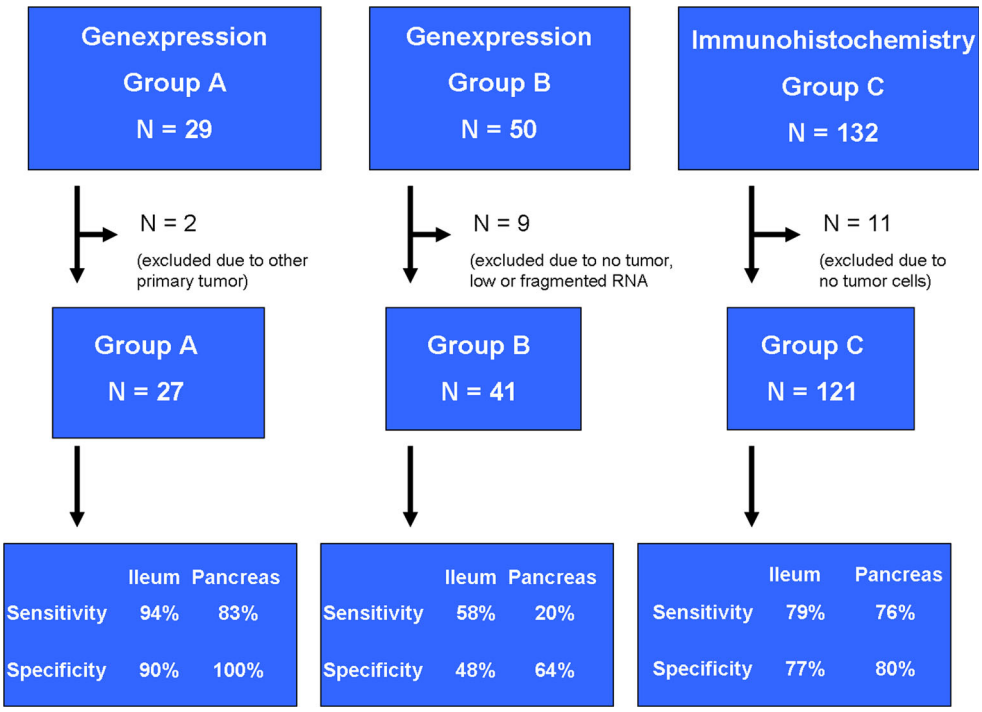

Fig. 1 Distribution of different investigation groups Group A: Specimen of the Zentralklinik Bad Berka, Group B: Specimen of the Institute of Pathology, Munich; Group C: Immunohistochemistry group of the Institute of Pathology, Jena

in 10–25 % of the patients during lifetime. The prognosis of patients with a CUP syndrome is therefore poor. The median survival ranges from 3 to 11 months, the 1-year survival time from 25 to 40 % [1]. Histologically most carcinomas are adenocarcinomas (40–60 %), followed by undifferentiated carcinomas (20–30 %) and squamous carcinomas (15–20 %). Neuroendocrine small cell carcinomas account for 5 %, but among the undifferentiated neoplasms they are the most common ones [2].

Neuroendocrine tumors (NETs) are often metastasized at diagnosis [3–5]. The primary NET is occult in 30 % of the patients at presentation. This limits therapeutic options, since these often depend on the origin of the primary. Previous studies have shown that somatostatin receptor PET/CT detects unknown NET primaries in 59 % [6]. However, even by using this sensitive technique, primaries could not be localized in more than 1/3 of patients [6]. In carcinomas of breast and colorectal origin as well as lymphomas it has been observed that the various tumor manifestations show striking similarities in their genetic profiles [7–9]. This was also reported in pancreatic NETs by Capurso et al. [10]. and ileal and pancreatic NETs by our group [11]. The findings have raised the question whether genetic signatures of NET metastases could help to identify the primary lesions, particularly, in case of liver metastases. Hainsworth et al. found in a prospective study that the detection of primary tumors by gene expression analysis (92-gene-assay) enabled a site-specific treatment that resulted in a prolonged overall survival [12]. In a pilot study using cDNA microarray technology, we identified three genes, namely *CD302, PPWD1, ABHD14B* whose expression profiles correlated with certain NET primaries [10]. Downregulation of *CD302* and *PPWD1* indicated the pancreas as site of the primary, whereas an upregulation of *ABHD14B* was associated with a primary of the stomach and an upregulation of *CD302* with a primary of the small intestine. Here we blindly examined the gene and protein expression of the three gene classifiers in abdominal NET metastases in order to predict the location of the respective primary tumor. The analysis was performed in three independent cohorts (Fig. 1).

Materials and methods

Tissue samples from NET-metastases were obtained during surgery, immediately frozen and stored by −80 °C in tumor tissue banks. In group A the tissue samples came from 29 metastases of 17 NET-patients and were provided by the Department of General and Visceral Surgery, Zentralklinik Bad Berka, Germany. In group B the specimens came from 50 NET-metastases and were provided by the Department of Pathology, Technical University of Munich, Germany. The frozen tissue samples were cut into 10 µm thick sections and the first section was stained with haematoxylin and eosin (HE) for histological evaluation. For RNA-isolation the tumor tissue from 10 to 20 unstained tumor sections were microdissected with the help of a laser-based microscope (Microbeam C; Zeiss) and collected in a minitube [13, 14]. Tissue from 5 metastases was

excluded from further analysis, because it made up less than 40 % of the total tissue of the section (Fig. 1).

3-Gene expression analysis

The One-Color Microarray-based Gene Expression Analysis was performed with the Human Genome Quick Amp Labeling Kit according to the manufacturer's instructions (www.agilent.com, protocol vs. 5.7, March 2008), as previously described [11]. RNA was isolated from the tumor tissue pieces using the RNeasy Mini Kit from Qiagen. RNA amount, purity and quality were checked with Nanodrop and gelelectrophorese. The sample input RNA varied between 50 ng and 1 µg. To amplify the target material and to generate fluorescent cDNA (complimentary DNA) simultaneously the Quick Amp Labeling Kit from Agilent was used. T7 RNA polymerase incorporates cyane 3-labeled CTP. Amplified and labeled cDNA samples were purified with the RNeasy Mini Kit from Qiagen. The cDNA and Cyane 3 dye concentrations were then measured using the NanoDrop ND-1000 UV–VIS Spectrometer vs. 3.2.1. The cDNA was hybridized to the 60 m length oligonucleotide probes on the 44 K microarray-surface. After 17 h of hybridization in a rotating oven (Agilent Technologies) at 65 °C and 10 rpm the microarrays were washed and scanned at 5 µm resolution with an Agilent G2565CA scanner. The raw data (tiff-Images) were extracted with Feature Extraction vs. 10.5.1.1. (Agilent Technologies) and analyzed with the GeneSpring GX vs. 10.0.2 (Agilent Technologies). The hybridization quality was checked for spatial artifacts with QC-Reports.

Statistical analysis of gene data

The row data were individually standardized by the quantile method (and by shift to 75 percentile) and background correction was done by using baseline transformation: median of all samples (median polishing), which results in log2 scale transformed data. To ensure a Gaussian-like distribution and to control the quality, the data were checked by using histograms, matrix plots, BoxWhisker Plots, Correlation Plots, Quality Control Metrics-Plot and by 3D PCA (Principal Component Analysis) Scores. The statistical analysis was done with ANOVA, with none post hoc test. For the *P* value computation the asymptomatically method and for *P* value correction the Benjamin Hochberg FDR (=false discovery rate) were chosen. Fold Change analysis was performed on the 1,760 selected genes which had an FDR corrected *P* value between 0.001 and 0.05. The Fold Change cut off was set by $\geq$10 which genes with Fold Change $\geq$10 were hierarchically clustered on Entities and Conditions by using Eucledian Distance Metric and Centroid Linkage Rule.

Table 1 Patient characteristics with neuroendocrine tumors and carcinoma (group A)

Patient	Sex	Localisation of metastases	TNM status	Karnofsky score (%)	KI-67-index (%)
1	F	Liver, peritoneum	T4N2M1	80	1
2	F	Liver, peritoneum	T4N2M1	80	2–5
3	M	Liver, lymph nodes	TxN2M1	80	5
4	F	liver	TxNxM1	90	1
5	M	Liver, lymph nodes	T3N1M1	n.d	2–5
6	M	Peritoneum	TxN0M1	80	40
7	F	Liver, 2 × mesentery	T3N0M1	80	15
8	F	Liver, lymph nodes	TxNxM1	90	2
9	M	Liver	T3N1M1	90	60
10	F	Lymph nodes	T2N1M0	90	2
11	F	Liver	TxNxM1	70	30
12	F	Liver, ovary, peritoneum	T3N1M1	90–100	1
13	M	Liver, lymph nodes	T3N1M1	90	2–5
14	M	Liver	T1NxM1	90	1
15	M	Lymph nodes	T4N1M0	90	5
16	F	2 × liver	TxNxM1	90–100	20–30
17	F	Liver, lymph nodes	T3N1M1	80–90	1

In a next step the hierarchical gene expression cluster of each metastasis was blindly compared with a previously established gene expression cluster database [11] containing the gene expression clusters of primary tumors from the ileum, stomach and pancreas. The results of the comparison were subsequently matched with the known clinical and pathological data. The specificity and sensitivity were statistically evaluated.

Validation of the expression profiles in group A and B

The study material of group A with its main biological and histologic features is listed in Table 1. From patient no. 50, three metastases from liver, ovary and peritoneum, respectively, were examined. From patient no. 55, two metastases of the liver were individually examined. The study material of group B consisted of 41 tumor samples because nine samples contained either no histologically identified tumor tissue or it was not possible to isolate quantitatively or qualitatively sufficient RNA (Table 2). Thirty-nine percent of the tumor samples were obtained between 1991 and 1999, 29 % between 2000 and 2005, and 32 % after 2006 (Table 2).

Table 2 Patient characteristics with neuroendocrine tumors and carcinoma (group B)

Tumor sample	Year of removal	RNA concentration (ng/µl)	Examined tissue	Localization of primary tumor
11076/91	1991	158.01	MTS	Stomach
11516/06	2006	49.61	MTS	Colon
12696/00	2000	276.45	MTS	Rectum
1300/01	2001	144.33	MTS	Pancreas
13300/10	2001	84.51	MTS	Pancreas
14065/06	2006	176.00	MTS	Colon
14291/00	2000	119.91	MTS	Pancreas
14504/00	2000	265.27	MTS	Pancreas
15019/02	2002	47.42	MTS	Pancreas
15153/10	2010	121.93	MTS	Pancreas
16368/96	1996	175.08	MTS	Duodenum
16804/03	2003	111.91	MTS	Ileum
1726/03	2003	52.75	MTS	Ileum
1861/11	2011	159.00	MTS	Ileum
19517/08	2008	94.88	MTS	Pancreas
19649/10	2010	79.78	MTS	Stomach
20018/97	1997	203.79	MTS	Pancreas
21324/10	2010	123.77	MTS	Pancreas
21960/99	1999	205.2	MTS	Colon
22194/10	2010	70.81	MTS	Pancreas
22666/99	1999	92.67	MTS	Ileum
23125/10	2010	74.44	MTS	Pancreas
23790/10	2010	493.87	MTS	Pancreas
24222/10	2010	91.84	MTS	Pancreas
24588/01	2001	172.8	MTS	Pancreas
24891/01	2001	164.85	MTS	Colon
26948/07	2007	81.66	MTS	Duodenum
2921/99	1999	12.53	MTS	Duodenum
3165/03	2003	72.02	MTS	Ileum
3228/96	1996	93.88	MTS	Ileum
3779/94	1994	3.38	MTS	Ileum
4201/98	1998	46.91	MTS	Stomach
5227/97	1997	239.63	MTS	Stomach
5460/09	2009	41.32	MTS	Pancreas
6463/08	2008	33.35	MTS	Colon
7596/96	1996	147.49	MTS	Colon
8118/99	1999	58.5	MTS	Duodenum
8631/97	1997	76.43	MTS	Stomach
9132/98	1998	43.65	MTS	Ileum
9263/97	1997	22.76	MTS	Ileum
9616/95	1995	13.33	MTS	Ileum

Immunohistochemical staining of tissue microarrays (TMAs)

Tissue microarrays (TMAs) were generated from archive tissue blocks according to known criteria. Paraffin sections from TMA blocks were stained immunohistochemically with antibodies against the proteins of *CD302* (1:500, Santa Cruz Biotechnology® Inc., USA), *PPWD1* (1:50, Santa Cruz Biotechnology® Inc., USA) and *ABHD14B* (1:200, Santa Cruz Biotechnology® Inc., USA) at the Institute for Pathology of the University Hospital Jena, Germany [15]. Of the 125 tumor samples 148 could be evaluated (Table 3). The staining intensity was scored as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

Table 3 Origin and number of immunohistochemically examined tumor specimens (group C)

Number of specimen	125
Primary tumors (total)	51
Pancreas	28
Ileum	18
Stomach	5
Metastases (total)	74
Colon/Rectum	15
Appendix	6
Liver	25
Peritoneum	6
Different	11
Unknown	11

Scores of 0/1 were considered negative, scores of 2/3 as positive. The immunohistochemical data of the stomach were not included into the analysis because there were only 6 primary specimens, of which 2 had no tumor cells. The statistical analysis was made with excel 2010, Office 14 (Microsoft Inc., Redmond, USA) and SPSS, version 11, (SPSS Inc. Chicago, USA).

Statistical methods for TMA analysis

For each of the three tumor entities pancreas/ileum/other a logistic regression with the dichotomous outcome "entity present yes/no" was estimated. In each case the immunohistochemical stainings of CD302, PPWD1 and ABHD14B were dichotomized into negative (weak (grade 1) or no expression (grade 0)) and positive (high (grade 3) or moderate (grade 2)). These three dichotomous variables were used as predictive variables in the logistic regression models. Tentatively, all two- and three-way interactions of these were added, using the likelihood-ratio test all insignificant interactions and variables were excluded from the model (Backward elimination). For the three resulting models a receiver operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was estimated to determine the accuracy of the model. Marginal means with 95 % confidence intervals (CI) as well as sensitivity and specificity were calculated

for all remaining significant combinations of the dichotomized immunohistochemical stainings. In addition to visualize the pathway of decision a decision tree for the combined three-stage outcome pancreas/ileum/other were estimated using the R package party [16].

All results presented are based on available case analysis ($n = 107$). Nominal P values are reported without correction for multiplicity. P values <0.05, two sided were considered significant. All analyses were conducted using Stata 13.1, STATA corporation, college station, Texas, US and the statistical software R 3.0.2 [17].

Results

Validation of 3-Gene expression analysis

We previously demonstrated in a gene expression study in gastroenteropancreatic NETs that the three genes *CD302, PPWD1* and *ABHD14B* were differentiately expressed in correlation with the localization of NET primaries in either the ileum, pancreas or stomach [11]. Here, we conducted two studies on probes from 70 NET metastases (study group A: $n = 29$, study group B: $n = 41$) to blindly validate the three gene classifiers. The study was deblinded regarding the origin of the metastases after reading the genetic profile of each tumor and predicting the origin of the primary.

In group A, in which 27/29 metastases could be evaluated, we were able to determine the origin of the primary in the ileum with a specificity of 90 % and a sensitivity of 94 %, and in the pancreas with a specificity of 100 % and a sensitivity of 83 % (Fig. 1). In the two cases that did not match any of the profiles typical for primaries of either in the ileum, stomach or pancreas, one (with a peritoneal metastasis) was found to have the primary in the appendix, while the other (with a liver metastasis) had the primary in the rectum.

In group B, the prediction of the tumor origin in the ileum was possible with a specificity of 48 % and a sensitivity of 58 % (5). For the pancreas the specificity was 64 % and the sensitivity 20 % (Fig. 1). The evaluation of group A and B together resulted in a specificity of 61 % and a sensitivity of 75 % for the ileum, and a specificity of 80 % and sensitivity of 38 % for the pancreas.

Validation by immunohistochemistry

Figures 2, 3, 4 show the immunohistochemical stainings of CD302 and PPWD1 with either high (grade 3), moderate (grade 2), weak (grade 1) or no expression (grade 0) (Figs. 2, 3, 4).

CD302 was expressed in the cytoplasma of the tumor cells of the metastases, if the corresponding primary was localized in the ileum. Fifty-four percent (18/33) of the tumor metastases of ileal origin stained grade 3 and 30 % (10/33) grade 2. Fifteen percent (5/33) of the "ileum samples" were classified as negative (grade 1 and 0). Thus, 84 % (28/33) of tumor samples from ileum primaries could be correctly assigned; 15 % (5/33) were regarded as false-negative (Table 4).

Downregulation of the expression of PPWD1 in NET metastases indicated the pancreas as site of the primary (Fig. 5). Tumor samples negative for CD302 were also negative for PPWD1 in 29 % (13/45) and showed a weak staining in 47 % (21/45). Altogether 76 % (34/45 pancreas specimen) were classified negatively. Only 24 % (11/45) of pancreatic specimens revealed a grade 2 positive staining.

In total, the origin of the ileal or pancreatic primary tumors were immunohistochemically correctly determined in 80 % (AUC 80 %) of the examined tumor samples in the TMAs (Table 4, Fig. 6).

Discussion

Patients with NET often present in an advanced stage with an unknown primary [5]. The recently updated guidelines of the European Neuroendocrine Tumor Society (ENETS) recommend the resection of the primary in case of a metastasized NET of the gastroenteropancreatic system, since it improves the patients prognosis. The identification of the primary is therefore necessary for an optimal therapy. The most successful diagnostic method for NET localizing and staging, with an accuracy of up to 98 %, is PET/CT using ^{68}Gallium [18–20]. However, in case of a CUP-NET syndrome the detection rate of the primary is clearly lower. Prasad et al. determined an accuracy of 59 % for ^{68}Ga-DOTA-NOC PET/CT whereas ^{111}In-OctreoScan showed a detection rate of only 39 % [6, 21]. In a surgical exploration study, Begum et al. successfully detected and removed NET primaries in 50 % of cases, which were mainly located in the midgut [22].

Gene expression profiling has already been used for the detection of primary carcinoma of the lung, breast and colon. In these malignancies, the identification of the primary had an impact on therapy leading to prolonged survival (12.5 vs 9.1 months) [12, 23]. Primary NETs of the ileum, pancreas or bile duct are much more difficult to detect [8, 9, 11, 24], and established gene expression assays or data based on such an analysis are so far not available in this condition. All known gene classifiers are multiclass classifiers which need the differential expression of at least ten genes in the metastasis for allocating the primary [23, 25–27] and those patterns have so far not been identified in NETs. Therefore, the development of a specific diagnosis system for NET patients with CUP syndrome is needed.

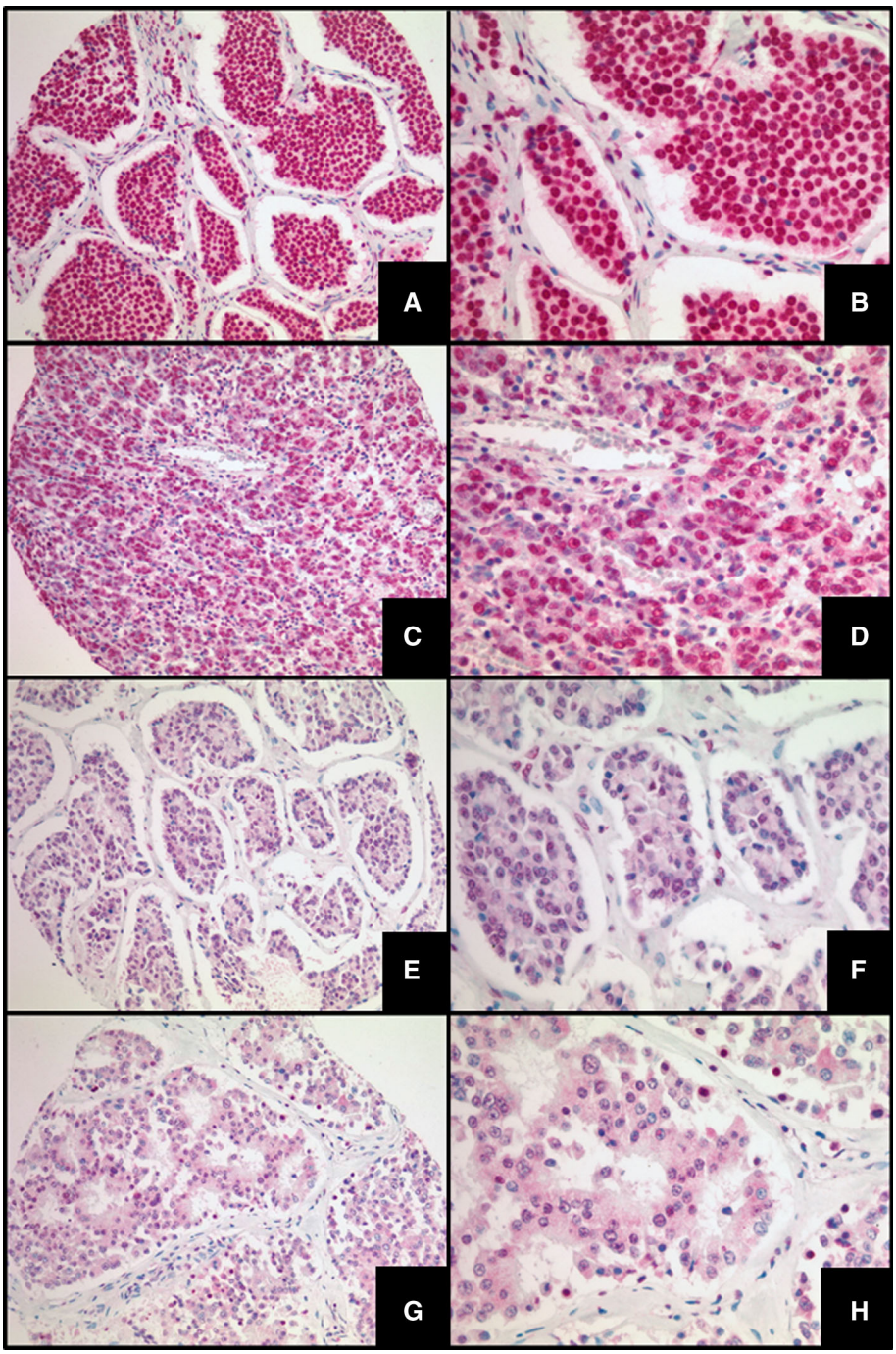

Fig. 2 Immunohistochemistry for CD302 was carried out on tissue microarrays with 148 neuroendocrine tumor samples. **a** (× 20) shows a very high expression of CD302 in the nucleus of the tumor cells (expression level 3). **b** (× 40) shows the same tumor. **c** (× 20) and **d** (× 40) show a neuroendocrine tumor with a lower expression level of CD302 (level 2). In **e** (× 20) and **f** (× 40) is shown a tumor exemplary for expression level 1. In **g** (× 20) and **h** (× 40) no nuclear expression of CD302 is detectable (level 0)

In a pilot study, we described three genes whose expression enabled us to assign NET metastases to their respective primaries in the ileum, pancreas or stomach [11]. The aim of this study was to validate these molecular classifiers under blinded conditions at the gene and protein level. For the gene expression analysis we evaluated two groups (A and B) of tumor specimens which were collected in different institutions and stored over different time periods. In group A, the origin of the primaries, either in the ileum or the pancreas, was predicted with a sensitivity of 94 % and 83 %, and a specificity of 90 % and 100 %, respectively. Of statistical reasons it was not possible to evaluate the stomach as origin of an unknown primary, because of insufficient test material. In group B, the specificity for both, the ileum and pancreas, was only 48 % and 64 %, and the sensitivity 58 % and 20 %, respectively. The reason for this discrepancy between the data of the two groups was the lower tissue quality of the samples in group B. Most of the tumor samples of this group dated back till 1991, while only 32 % of the tumor samples were removed in the same period (2007–2010) as the samples of group A. The

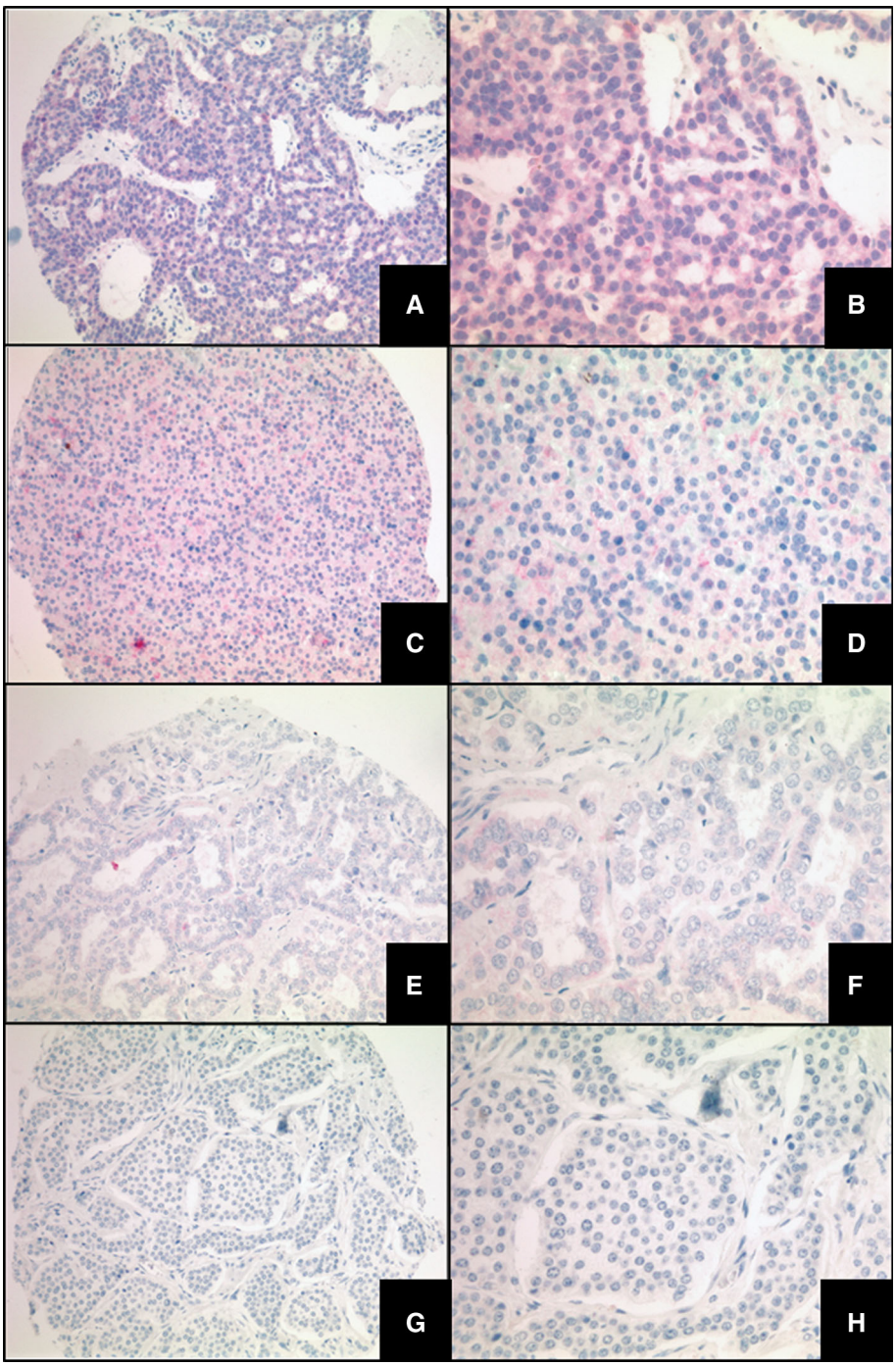

Fig. 3 Immunohistochemistry for PPWD1 was carried out on tissue microarrays with 148 neuroendocrine tumor samples. **a** (× 20) and **b** (× 40) show a very high expression of PPWD1 in cytoplasm of the tumor cells, (expression level 3). **c** (× 20) and **d** (× 40) show a neuroendocrine tumor with lower expression of PPWD1 (level 2). In **e** (× 20) and **f** (× 40) is shown an exemplary tumor for expression level. In **g** (× 20) and **h** (× 40) no cytoplasmic expression of PPWD1 is shown (level 0)

storage for two decades, with probably freezing and defreezing episodes, had obviously led to a degradation of the RNA that "falsified" the expression data. Reliable gene expression analysis requires therefore well preserved frozen tumor tissue. This was also concluded in a study by Musella, who investigated the effects of tissue handling on gene expression. They demonstrated the impact of hypoxia and prolonged storage times on mRNA expression levels in different tumor entities [28].

Gene expression analysis requires frozen tissue that, however, in many cases is not available for examination. We therefore also studied the protein expression of the three gene classifiers, using immunohistochemistry (IHC) as validation method. TMAs composed of 132 NETs were screened for the expression of CD302, PPWD1 and ABHD14B using commercial antisera against these proteins. The analysis of ABHD14B was removed because there were only four valid primary tumors and no metastasis at all.

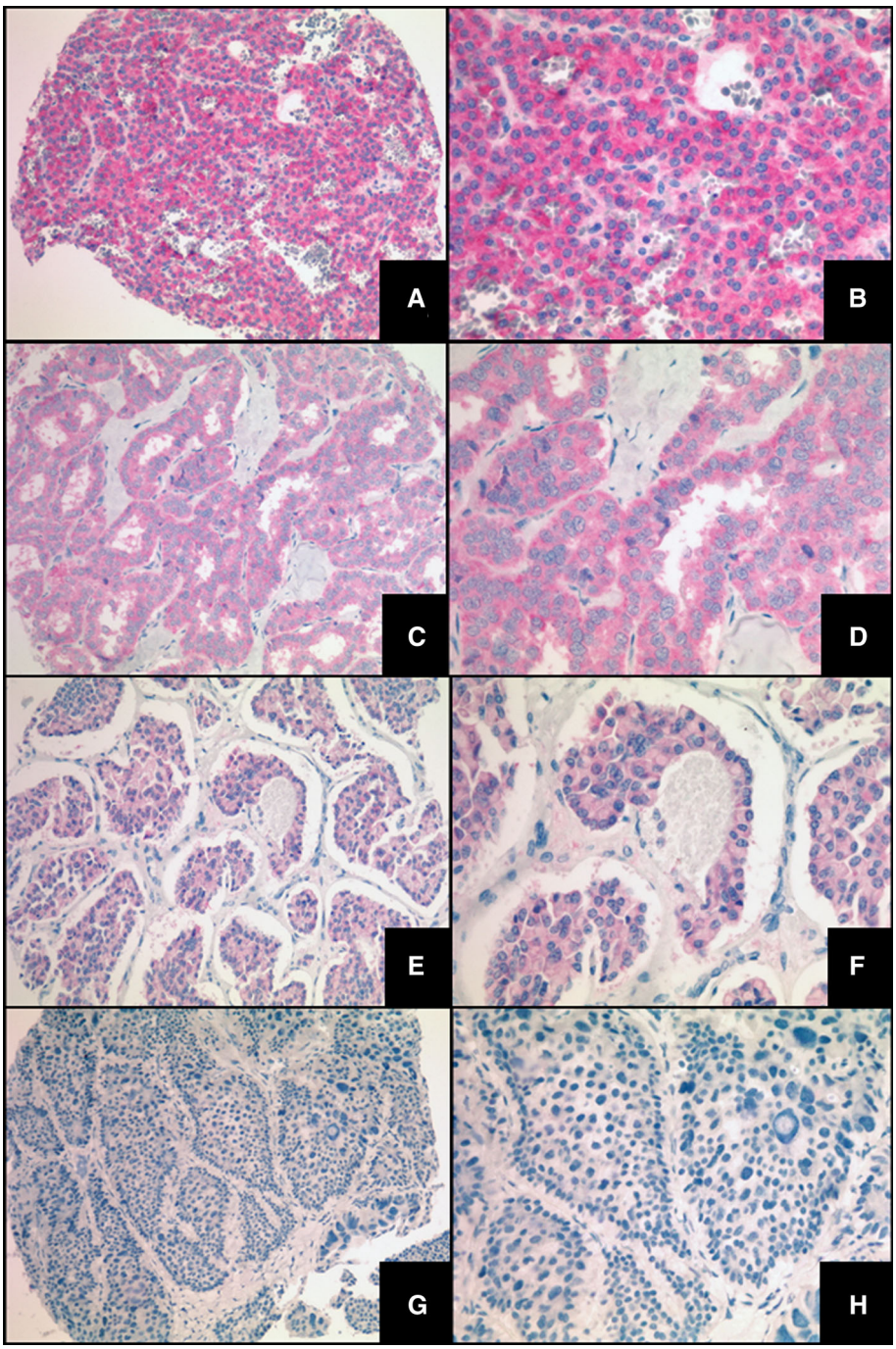

Fig. 4 Immunohistochemistry for ABHD14B was carried out on tissue microarrays with 148 neuroendocrine tumor samples. **a** (× 20) and **b** (× 40) show a very high expression of ABHD14B in cytoplasm of the tumor cells (expression level 3). **c** (× 20) and **d** (× 40) show a neuroendocrine tumor with lower expression of ABHD14B (level 2). In **e** (× 20) and **f** (× 40) is shown an exemplary tumor for expression level 1. In **g** (× 20) and **h** (× 40) no cytoplasmic expression of ABHD14B is shown (level 0)

Bubendorf et al. could show that the results of IHC examination on TMAs and on the corresponding stamp tissue are almost identical [29]. The disadvantage of the TMAs is that due to the small size of the assembled specimens some sections do not contain all original tumor samples, which can therefore not be evaluated. This was the case in 11 of our 132 tumor samples. In the remaining 121 tumors, the evaluation of the stainings revealed in total a sensitivity of the immunohistochemical analysis of the gene classifiers of 80 %. Only the genes PPWD1 and CD302 have to be analyzed for primary detection. Figures 5 and 6 demonstrate the AUC and the decision tree, which could be easily used in pathological daily routine. The discrepancy between the RNA data and the lower protein expression results may be explained by modifications at the translational level. A high regulated gene in one cell can still be associated with a low protein expression, if the mRNA copies are copied only once. The results of a gene expression analysis can therefore not totally be applied to the protein level. This implies that immunohistochemical analysis cannot completely replace

Table 4 Statistical evaluation of the immunohistochemical staining in neuroendocrine specimens

Tumor	PPWD1	CD302	*n*	Probability	95 % CI		*p* value	Sensitivity	Specificity	AUC	95 % CI	
					Lower bound	Upper bound					Lower bound	Upper bound
Ileum	Negative	Negative	2	0.04	0.00	0.10	0.099	1.00	0.00	0.804	0.722	0.885
		Positive	2	0.25	0.03	0.47	0.028	0.85	0.66			
	Positive	Negative	3	0.17	0.03	0.32	0.021	0.94	0.43			
		Positive	26	0.59	0.45	0.74	0.000	0.79	0.77			
Pancreas	Negative		34	0.77	0.65	0.90	0.000	0.76	0.80	0.797	0.719	0.876
	Positive		11	0.17	0.08	0.27	0.000	1.00	0.00			
Other	Negative	Negative	5	0.16	0.04	0.28	0.007	0.97	0.12	0.709	0.601	0.817
		Positive	1	0.05	0.00	0.11	0.109	1.00	0.00			
	Positive	Negative	12	0.57	0.37	0.78	0.000	0.41	0.90			
		Positive	11	0.27	0.14	0.40	0.000	0.79	0.49			

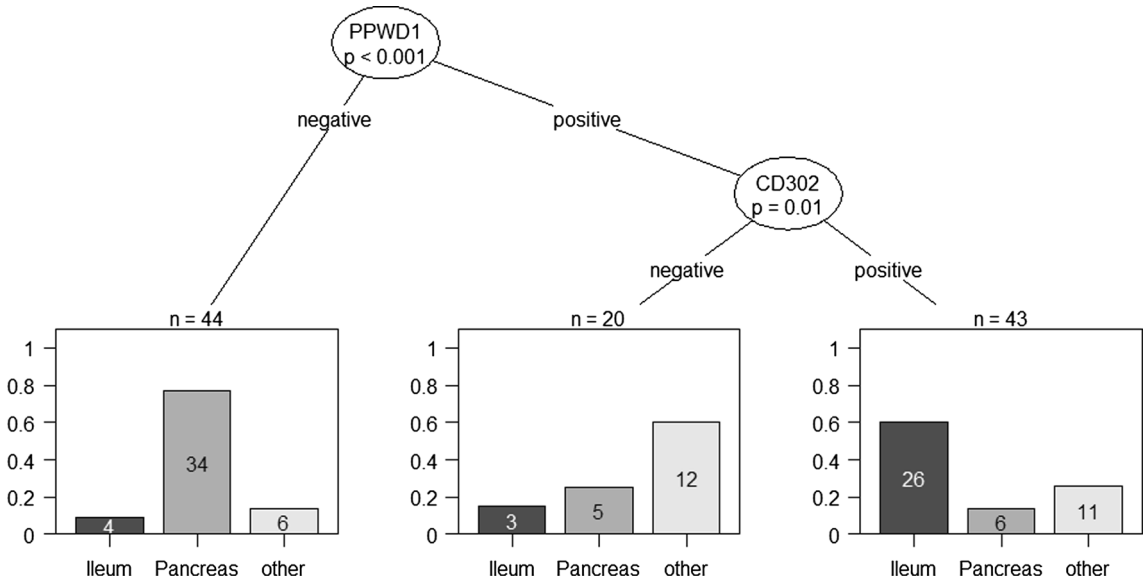

Fig. 5 Decision tree summarizes immunohistochemical results to detect pancreatic and ileal primaries

genetic analysis. However, because IHC is less cost-effective and time consuming and more easily to perform than gene analysis, it is the method of choice in routine work. Other markers that are used in localizing the primaries of NETs are PDX-1 and CDX-2. PDX-1 is positive in most pancreatic NETs (sensitivity 72 %, specificity 93 %), but is also positive in a number of non-NETs. CDX-2, in contrast to PDX1, has a high sensitivity (85 %) and specificity (98 %) for intestinal NETs [30] but without any other organ specificity. Furthermore, the human insulin gene enhancer-binding protein islet-1 (ISL1) can be used as a marker for NETs of pancreatic origin. The specificity however is limited by the fact that it also stains several extrapancreatic NETs such as medullary thyroid carcinoma (MTC) and pheochromocytoma [31]. Currently, there are no data available about the performance of our two-gene-classifier in metastasis of MTC or other extra-intestinal tumors. These questions have to be answered by further studies. But with an overall sensitivity of 80 % our immunohistochemical results are at least comparable to the best IHC markers which are currently used. CD302 as a marker for NETs localized in the ileum predicts these tumors in 84 %. The PPWD1 negativity, on the other hand, correlates closely with NETs of pancreatic origin. Due to the more frequent and simple application with comparable results to gene expression data the use of immunohistochemical analysis can be recommended.

In summary, the study shows that the expression of the two genes, *CD302 and PPWD1*, in liver metastases from NETs help to predict the origin of the primary, if it is

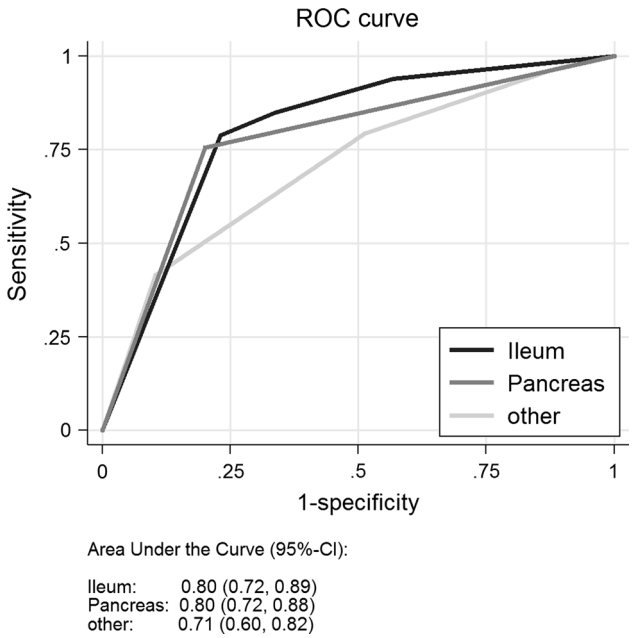

Fig. 6 ROC curve shows the results of the prediction of ileum- or pancreatic origin in immunohistochemistry

localized either in the ileum or pancreas. This two-gene-classifier can be applied additively for the search of the primary in neuroendocrine CUP-patients and enables of a successful application adequate tumor treatment.

Conflict of interest Kaemmerer D., Posorski N., von Eggeling F. and Hommann M. are coholders of a patent related to *CD302*, *PPWD1*, *ABHB14B*. No other conflicts of interest are disclosed regarding this manuscript.

References

1. Ettinger DS, Agulnik M, Cates JM et al (2011) Occult primary. J Natl Compr Cancer Netw 9(12):1358–1395
2. Pavlidis N, Pentheroudakis G (2010) Cancer of unknown primary site: 20 questions to be answered. Ann Oncol 21(Suppl 7):303–307
3. Morris GJ, Greco FA, Hainsworth JD et al (2010) Cancer of unknown primary site. Semin Oncol 37(2):71–79
4. Balaker AE, Abemayor E, Elashoff D et al (2012) Cancer of unknown primary: does treatment modality make a difference? Laryngoscope 122(6):1279–1282
5. Stoyianni A, Pentheroudakis G, Pavlidis N (2011) Neuroendocrine carcinoma of unknown primary: a systematic review of the literature and a comparative study with other neuroendocrine tumors. Cancer Treat Rev 37(5):358–365
6. Prasad V, Ambrosini V, Hommann M et al (2010) Detection of unknown primary neuroendocrine tumours (CUP-NET) using (68) Ga-DOTA-NOC receptor PET/CT. Eur J Nucl Med Mol Imaging 37(1):67–77
7. Melle C, Ernst G, Schimmel B et al (2008) Colon-derived liver metastasis, colorectal carcinoma, and hepatocellular carcinoma can be discriminated by the Ca(2+)-binding proteins S100A6 and S100A11. PLoS ONE 3(12):e3767
8. Giordano TJ, Shedden KA, Schwartz DR et al (2001) Organ-specific molecular classification of primary lung, colon, and ovarian adenocarcinomas using gene expression profiles. Am J Pathol 159(4):1231–1238
9. Horlings HM, van Laar RK, Kerst JM et al (2008) Gene expression profiling to identify the histogenetic origin of metastatic adenocarcinomas of unknown primary. J Clin Oncol 26(27):4435–4441
10. Capurso G, Lattimore S, Crnogorac-Jurcevic T et al (2006) Gene expression profiles of progressive pancreatic endocrine tumours and their liver metastases reveal potential novel markers and therapeutic targets. Endocr Relat Cancer 13(2):541–558
11. Posorski N, Kaemmerer D, Ernst G et al (2011) Localization of sporadic neuroendocrine tumors by gene expression analysis of their metastases. Clin Exp Metastasis 28(7):637–647
12. Hainsworth JD, Rubin MS, Spigel DR et al (2013) Molecular gene expression profiling to predict the tissue of origin and direct site-specific therapy in patients with carcinoma of unknown primary site: a prospective trial of the Sarah Cannon research institute. J Clin Oncol 31(2):217–223
13. Melle C, Ernst G, Schimmel B et al (2003) Biomarker discovery and identification in laser microdissected head and neck squamous cell carcinoma with ProteinChip technology, two-dimensional gel electrophoresis, tandem mass spectrometry, and immunohistochemistry. Mol Cell Proteomics 2(7):443–452
14. Melle C, Ernst G, Schimmel B et al (2004) A technical triade for proteomic identification and characterization of cancer biomarkers. Cancer Res 64(12):4099–4104
15. Knosel T, Chen Y, Altendorf-Hofmann A et al (2012) High KIT and PDGFRA are associated with shorter patients survival in gastroenteropancreatic neuroendocrine tumors, but mutations are a rare event. J Cancer Res Clin Oncol 138(3):397–403
16. Torsten Hothorn KHaAZ (2006) Unbiased recursive partitioning: a conditional inference framework. J Comput Graph Stat 15(3):651–674
17. R Development Core Team (2010) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing
18. Ambrosini V, Campana D, Nanni C et al (2012) Is (6) (8)Ga-DOTA-NOC PET/CT indicated in patients with clinical, biochemical or radiological suspicion of neuroendocrine tumour? Eur J Nucl Med Mol Imaging 39(8):1278–1283
19. Baum RP, Prasad V, Hommann M et al (2008) Receptor PET/CT imaging of neuroendocrine tumors. Recent Results Cancer Res 170:225–242
20. Baum RP, Kulkarni HR, Carreras C (2012) Peptides and receptors in image-guided therapy: theranostics for neuroendocrine neoplasms. Semin Nucl Med 42(3):190–207
21. Savelli G, Lucignani G, Seregni E et al (2004) Feasibility of somatostatin receptor scintigraphy in the detection of occult primary gastro-entero-pancreatic (GEP) neuroendocrine tumours. Nucl Med Commun 25(5):445–449
22. Begum N, Hubold C, Buchmann I et al (2013) Diagnostics and therapy for neuroendocrine neoplasia of an unknown primary–a plea for open exploration. Zentralbl Chir 139(3):284–291
23. Bridgewater J, van Laar R, Floore A et al (2008) Gene expression profiling may improve diagnosis in patients with carcinoma of unknown primary. Br J Cancer 98(8):1425–1430
24. Shedden K, Taylor JM, Enkemann SA et al (2008) Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. Nat Med 14(8):822–827
25. Ramaswamy S, Tamayo P, Rifkin R et al (2001) Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci USA 98(26):15149–15154
26. Alizadeh AA, Ross DT, Perou CM et al (2001) Towards a novel classification of human malignancies based on gene expression patterns. J Pathol 195(1):41–52

27. Su AI, Welsh JB, Sapinoso LM et al (2001) Molecular classification of human carcinomas by use of gene expression signatures. Cancer Res 61(20):7388–7393
28. Musella V, Verderio P, Reid JF et al (2013) Effects of warm ischemic time on gene expression profiling in colorectal cancer tissues and normal mucosa. PLoS ONE 8(1):e53406
29. Bubendorf L, Nocito A, Moch H et al (2001) Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 195(1):72–79
30. Chan ES, Alexander J, Swanson PE et al (2012) PDX-1, CDX-2, TTF-1, and CK7: a reliable immunohistochemical panel for pancreatic neuroendocrine neoplasms. Am J Surg Pathol 36(5):737–743
31. Agaimy A, Erlenbach-Wunsch K, Konukiewitz B et al (2013) ISL1 expression is not restricted to pancreatic well-differentiated neuroendocrine neoplasms, but is also commonly found in well and poorly differentiated neuroendocrine neoplasms of extrapancreatic origin. Mod Pathol 26(7):995–1003