Review paper

Using large-scale tissue microarray resources for the identification and evaluation of old, new, and novel targets for surgical pathology

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Immunohistochemistry (IHC) is essential in diagnostic pathology, but antibody reliability is often limited by insufficient validation and high variability between clones. Conventional validation approaches normally use only few tissues or cell lines which do not reflect tissue and cell type complexity. A high-throughput validation platform based on large-scale tissue microarrays (TMAs) was established to assess antibody specificity across 76 normal tissue types and to evaluate the diagnostic performance in > 15,000 tumours from > 130 entities. Comparative staining with independent antibodies on a full set of normal tissues enables robust detection of cross-reactivity. Comparative staining of critical tumour cohorts under different conditions enables protocol optimization to obtain the best possible sensitivity and specificity for the distinction of different tumour types. Large-scale tumour profiling studies on ≥ 15,000 cancers generated comprehensive datasets refining the diagnostic value of established markers (e.g., CK7/CK20, PLAP) and characterizing the impact of emerging diagnostic targets such as MTAP, CDH16, and CDH17. Furthermore, screening of over 6000 antibodies of previously unknown clinical significance identified various novel diagnostic markers, including CPA1, GAD2, and KDM6A, with high specificity for distinct tumour entities and/or neoplastic transformation. In conclusion, large-scale TMA-based validation improves antibody identification and characterization and provides a robust framework for integrating IHC into digital pathology solutions.

Key words: immunohistochemistry, antibody validation, tissue microarray, cancer, targeted therapy.

Introduction

Immunohistochemistry (IHC) is the most widely used auxiliary method in diagnostic pathology. It enables the identification and sometimes also quantification of proteins in cells and extracellular structures. Most commonly it is done with the intention to either classify cell types that are otherwise difficult to distinguish or to identify cells that are difficult to identify by morphology alone, such as small lymph node metastasis or lymph vessels within tumour tissue. The identification of the tissue of origin of malignant neoplasms is a major application for IHC in surgical pathology. This approach assumes that proteins that are expressed in non-neoplastic cells are often retained in neoplastic transformed cells. Unfortunately, there are, however, only a few cancer types that regularly express one or several proteins that are fully specific for the respective cancer type. These include, for example, prostate-specific antigen (PSA) and prostate-specific alkaline phosphatase (PSAP) for prostate cancer [1, 2], thyroglobulin for follicular cell-derived thyroid cancer [3], and carboxypeptidase A1 (CPA1) for pancreatic acinar cell carcinomas [4]. Most other proteins that are used for the characterisation of a cancer's origin are less specific. This is due to a paucity of cell type-specific proteins and because neo-expression of most proteins can occur – at variable frequency – in dedifferentiated neoplastic cells irrespective of their cell type of origin.

For the distinction of more than 1200 cancer types and subtypes, specialised pathology centres employ an arsenal of 100 to >250 different antibodies. Most antibodies used for tumour categorisation are not cancer-type specific, but the expected frequencies of positive staining results differ markedly between tumour entities considered in a given clinical setting. In an optimal world, every surgical pathologist would know for each antibody of his available arsenal the likelihood of staining positive in all these tumour types. It is unfortunate that this information cannot even be compiled from the literature for most antibodies because results of published studies are often highly discordant, especially for target proteins that were often subject to publication. For many diagnostically relevant target proteins, the rates of positivity describe range from very low to very high, and sometimes even 0–100% in several tumour types. As an example, compiled literature data on E-cadherin expression in combination with results from our own study are given in Figure 1. This diversity of data primarily results from differences in antibody properties, staining protocols, and criteria for staining interpretation. It must be understood that antibodies are produced in experimental animals that always generate a broad range of different antibodies if vaccinated with a target peptide or protein, and it is simply not possible for antibody manufacturers to easily select "the best" of these antibodies. Antibody validation by manufacturers is often limited to analyses of one or a few cell lines, some of which may have undergone a knock-out or forced overexpression of the target protein [5]. Considering the diversity of cell types in humans, this approach is obviously insufficient to rule out cross-reactivity with proteins that are not present in employed cell lines.

Antibody validation

To establish a portfolio of fully specific high-quality antibodies, we have developed a high-throughput antibody validation and characterisation pipeline based on the availability of a large-scale tissue microarray (TMA) repository. To assure the specificity of antibodies for IHC, the International Working Group of Antibody Validation (IWGAV) [6] has recommended comparing its staining results either with expression data obtained by another method or with immunostaining results obtained by an independent second antibody. We have adopted this approach and expanded the requirements by applying it to a near complete set of normal tissues. The regular use of 76 different normal tissue types (8 donors each) in a normal tissue TMA ensures that the vast majority of cell types from adult humans will be represented in each antibody validation process. It is thus likely that a near-complete spectrum of human proteins with a broad spectrum of posttranslational modifications will be evaluated for potential cross-reactivities. It is of note that more than 90% of antibodies that are labelled as "works on paraffin embedded tissues" and that were tested by our group have shown either overt cross-reactivities or very unfavourable signal-to-noise ratio. Cross-reactivities of antibodies are best discovered by a comparison of two or more antibodies on consecutive tissue sections. While all antibodies will normally stain their target proteins, antibody-specific cross-reactivities cause a distinct staining of structures that remain unstained by other antibodies to the same target. Examples of cross-reactivities of frequently used and/or CE-IVD-certified clones are given in Figure 2.

Antibody performance characterisation

Once an antibody has demonstrated optimal specificity and when optimal staining conditions have been determined, it is worthwhile to comprehensively characterise its utility as a diagnostic marker. For this purpose, it is useful to assess its staining behaviour in an as large as possible cohort of tumours of as many as possible different tumour types and subtypes. We typically examine > 15,000 cancers from > 130 tumour entities [7–29]. For logistical reasons, such a study can only be done in a TMA format. Besides economics, its unprecedented level of standardisation represents a key advantage of TMA studies.

In addition to parameters that can also be standardised in automated IHC staining systems, such as antibody concentration or incubation time and temperature, there are further soft parameters such as the slide age and the quantity of tissue analysed, which are difficult to standardise by other methods. The time between sectioning and staining of a tissue slide can greatly affect IHC results. A delay of as little as two weeks can result in a significant decrease of staining intensity for many antibodies [30]. Studies comparing TMA vs. whole section results have demonstrated that the likelihood of a positive IHC staining increases markedly with the quantity of tissue

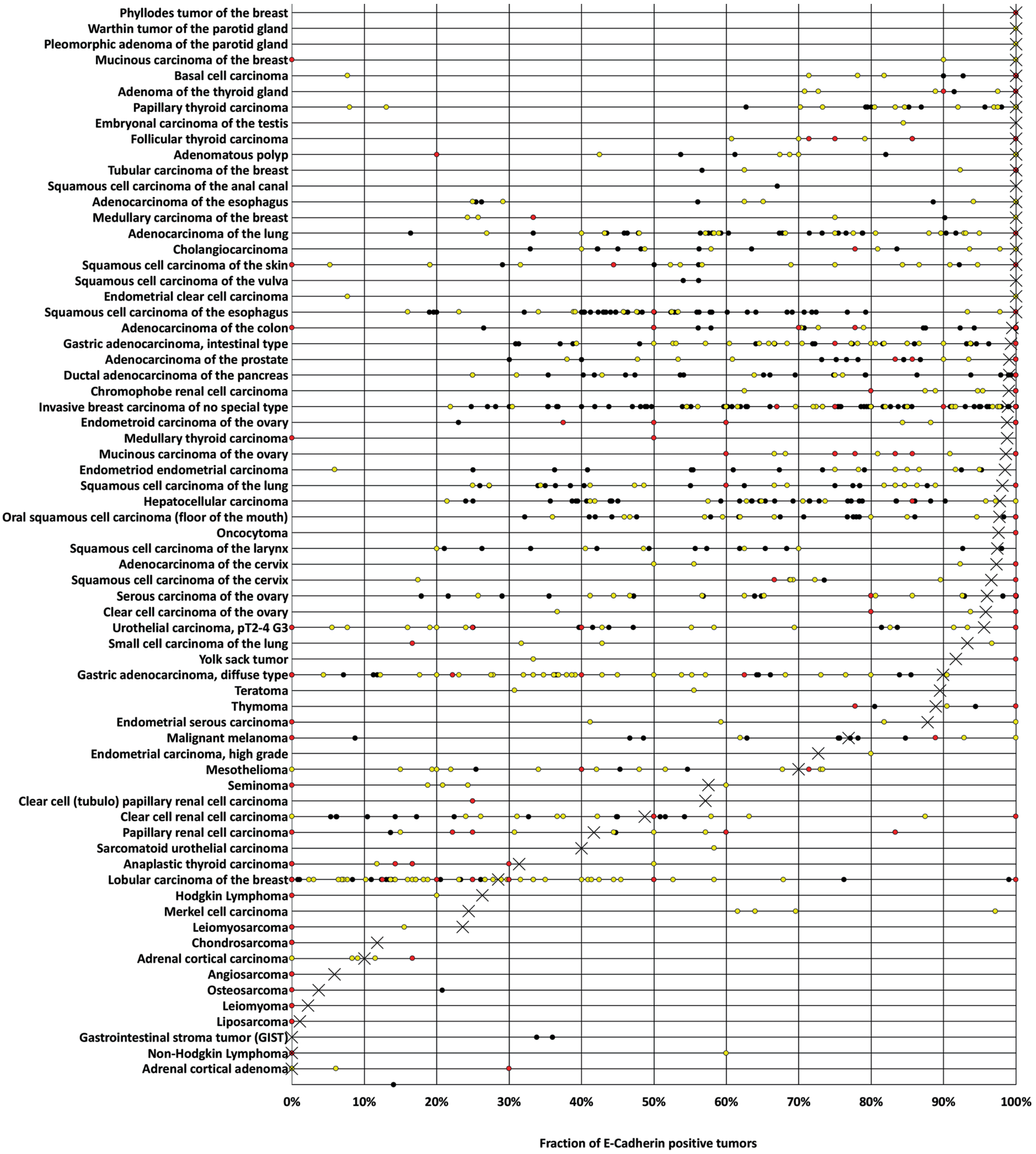

Figure 1. Graphical summary of E-cadherin immunohistochemistry data in published studies

Red symbols indicate studies including 2–10 cases, yellow symbols indicate studies with 11–50 cases, and black symbols represent studies comprising more than 50 cases. Figure taken from Burandt et al. {63}. An X indicates the results of the study from Burandt et al. {63}

analysed [31–34]. Authors have therefore suggested that TMAs should always contain multiple samples per tumour to increase representativity [31–33]. We advise against following this recommendation because it increases the workload and the use of tissue by at least a factor of 2 without significantly increasing the representativity for entire tumours. It must be recognised that even a 2.5-μm whole section containing a tumour area of 3 × 2 cm only represents 0.0023% of the mass of a tumour measuring 5 cm in diameter. Even more importantly, the quantity of tumour tissue analysed per patient is highly variable on whole sections (range: < 1 mm^2 to > 6 cm^2) while TMAs containing one sample of 0.6-mm tumour tissue per patient allow the analysis of a standardised tumour mass per patient. Tissue microarrays thus always offer a standardised representativity of IHC data for entire tumour masses. Accordingly, the only study comparing whole sections and TMAs for their suitability to detect prognostic parameters found clearly superior results for TMAs in a cohort of > 550 breast cancers for one out of three analysed markers [35] (Figure 3).

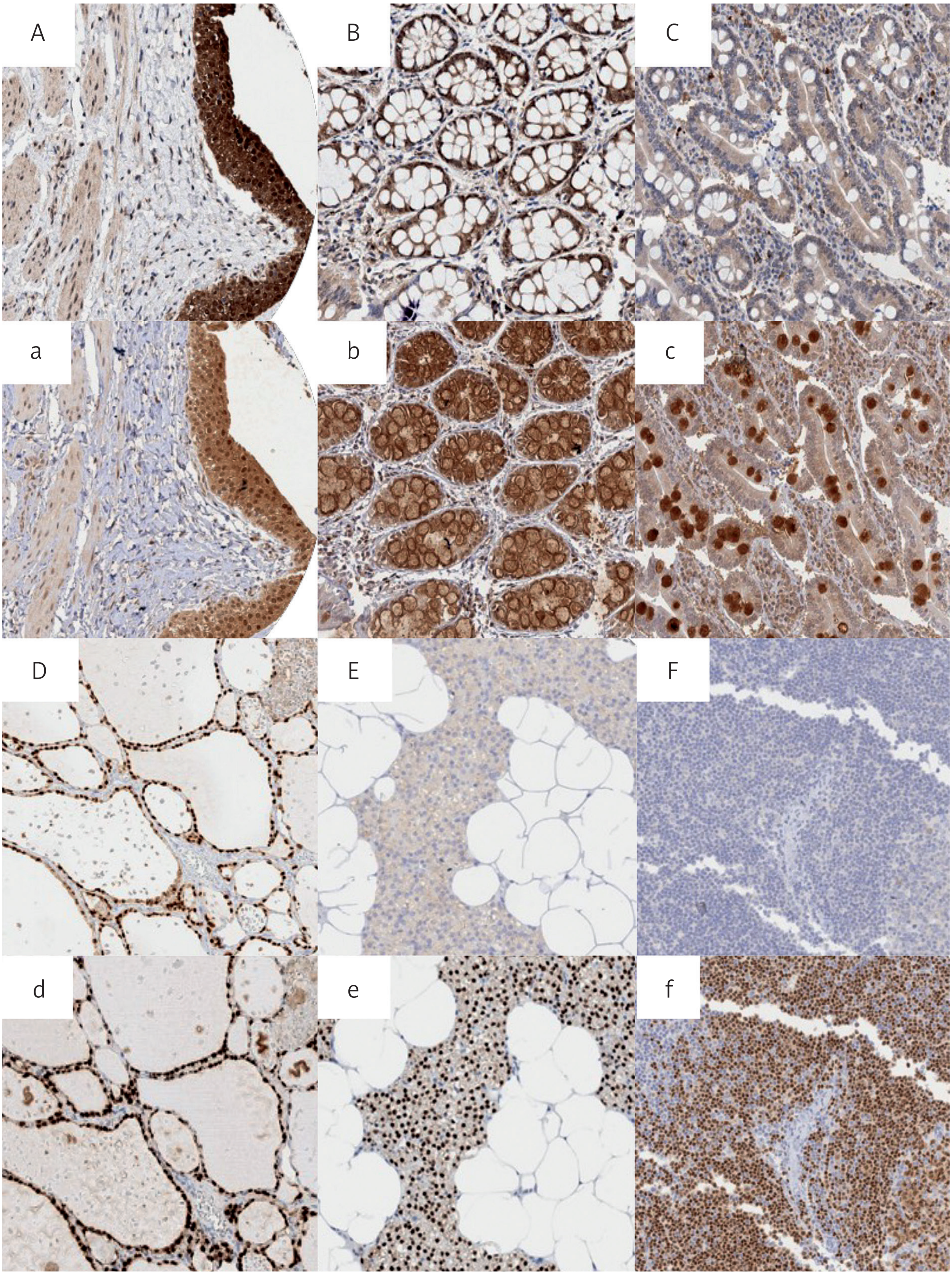

Figure 2. Examples of antibody cross-reactivity. **A–C**) Staining with methylthioadenosine phosphorylase (MTAP) antibody MSVA-741R in urothelium (**A**), colon (**B**) and duodenum (**C**). **a–c**) Staining with anti-MTAP clone 2G4 confirms all staining patterns observed by MSVA-741R but shows additional staining (cross reactivity) in goblet cells of the colon (**b**) and the duodenum (**c**). **D–F**) PAX8 MSVA-708R shows positive nuclear staining in thyroid gland cells (**D**), but does not stain parathyroid gland (**E**) and lymph node (**F**). **d–f**) Staining with PAX8 clone MRQ-50 confirms staining in thyroid gland (**d**) but shows additional staining (cross-reactivity) in cells of parathyroid gland (**e**) and lymph node (**f**)

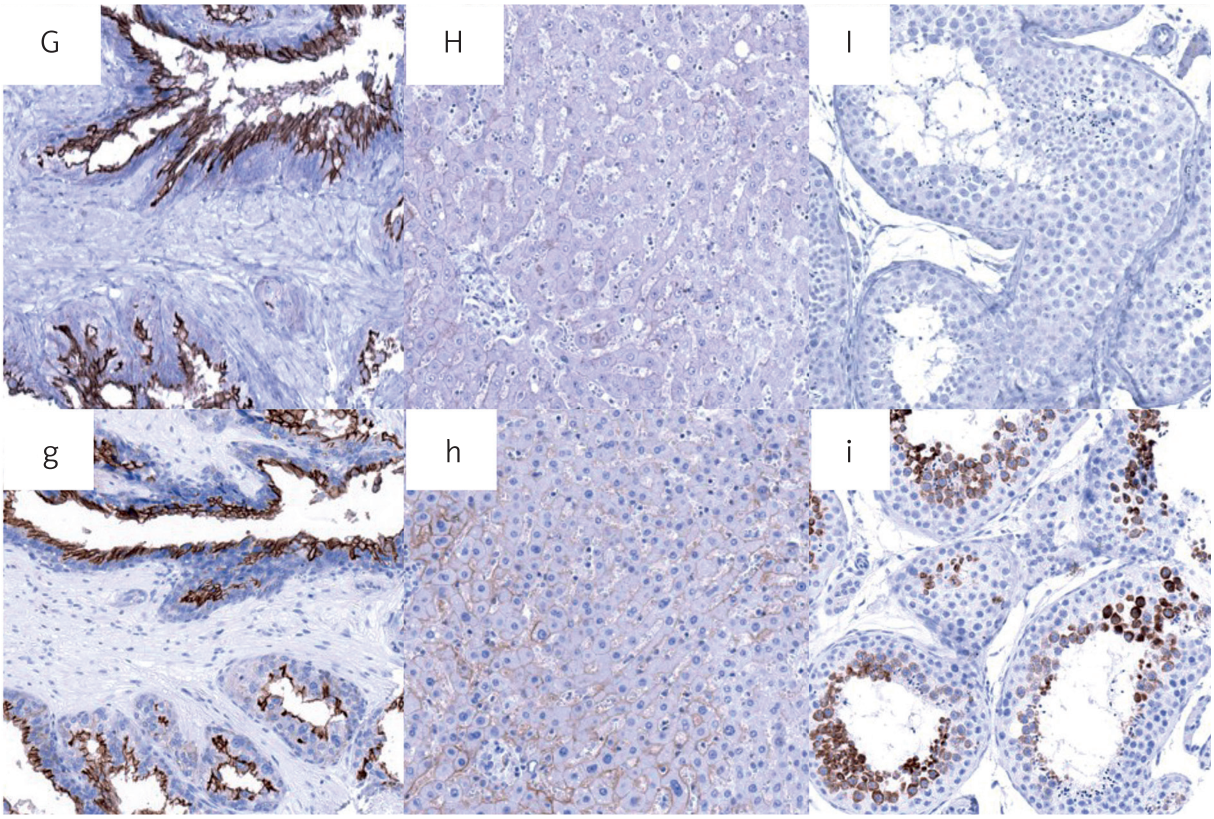

Figure 2. Cont. DOG1 staining by MSVA-201M reveals positivity in seminal vesicle epithelium (**G**), but is negative in liver (**H**) and testis (**I**). **g–i**) Staining with DOG1 clone SP31 confirms staining in the seminal vesicle (**g**) but shows additional staining (cross-reactivity) in liver (h) and testis (i)

The main result of each multi-tumour TMA study is a comprehensive list of tumour entities with data on the likelihood of each cancer type to stain positively with a certain antibody. In more than 50 studies we have recently contributed to a better characterisation of established diagnostic IHC target proteins [9, 29, 36–40], clarified the utility of more recently described diagnostic targets [11, 15, 16, 19, 41–43], and identified several new markers with considerable diagnostic utility [4, 17, 18, 43–46].

Better characterisation of old targets

The systematic and highly standardised evaluation of > 100 important tumour entities provided useful new data for many diagnostic markers that have been used for more than 30 years and for which dozens or hundreds of publications exist. For example, the joint analysis of CK20 and CK7, a frequently used combination of antibodies, enabled the dissection of their diagnostic utility both alone and in combination [40]. These data corroborated that CK20 is a useful marker for gastrointestinal, biliopancreatic (or urothelial) tumour origin, while the information provided by CK7 staining appeared to be less important due to its frequent positivity in virtually all epithelial neoplasms. Although CK7 positivity was found in only 8% of colorectal and 18% of prostatic adenocarcinomas, it must be admitted that more specific markers do currently exist to support a diagnosis of these cancers. Placental alkaline phosphatase (PLAP) is often *de novo* expressed in cells of germ cell neoplasms and is therefore employed as a marker for testicular cancer [47, 48]. Our analysis of more than 16,000 tumours from 131 different entities revealed, however, that strong PLAP expression can also be found in other clinically important cancers such as gastrointestinal and biliopancreatic cancers which can, similarly to testicular neoplasms, cause retroperitoneal lymph node metastases [36].

Early characterisation of new targets

Methylthioadenosine phosphorylase (MTAP), cadherin 16 (CDH16), and cadherin 17 (CDH17) are examples of newly proposed diagnostic antibody targets, which until now have been evaluated only in a limited number of indications [11, 15, 16]. In studies evaluating thousands of cancers, we were able to sharply delineate their diagnostic potential. MTAP is an enzyme needed for phosphorolysis of 5′-methylthioadenosine (MTA) in the methionine salvage pathway, which is highly expressed in most cell types. Because of the location of the MTAP gene in the immediate vicinity of CDKN2A, which is homozygously deleted in almost 10% of all tumours, MTAP is co-deleted in a large fraction of these cases. Homozygous deletion of the MTAP gene only occurs in neoplastic tis-

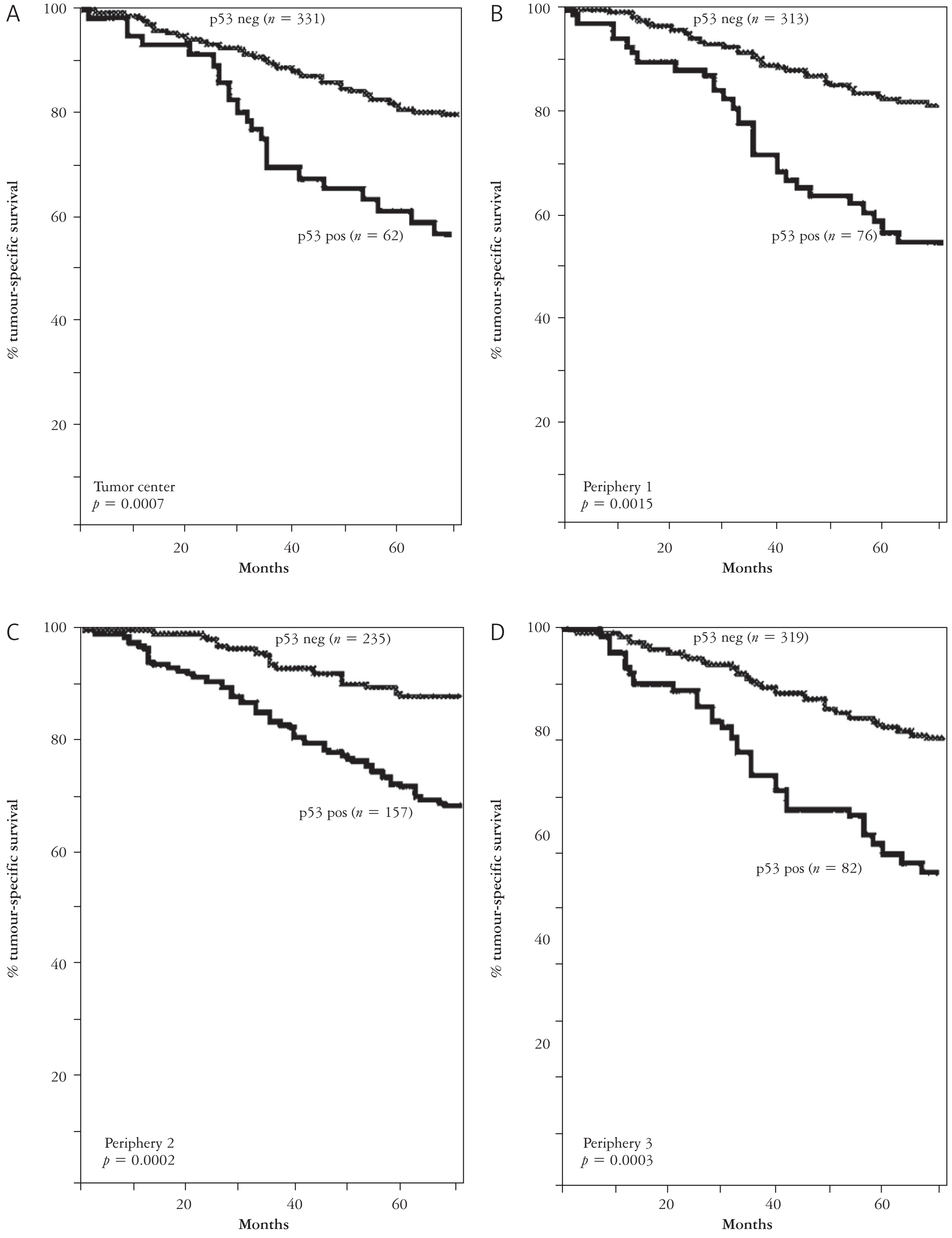

Figure 3. p53 immunostaining and prognosis in breast cancer. The association with tumour-specific survival is shown for p53 data obtained from one tissue microarray (TMA) spot from the tumour center (**A**), and three TMA spots from the tumour periphery (**B–D**)

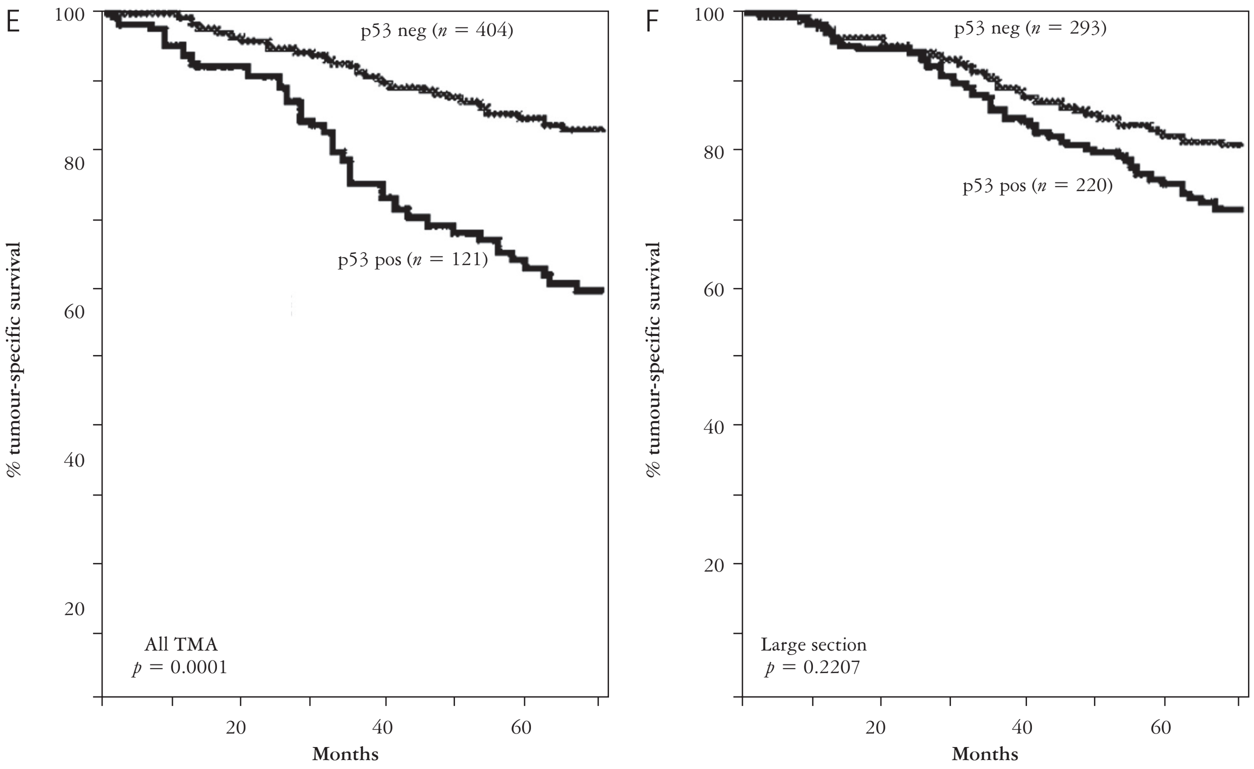

Figure 3. Cont. Association between tumour-specific survival and p53 data obtained from the combined analysis of all four TMAs (**E**), and from whole sections (**F**) of the donor blocks used for TMA manufacturing

Figure modified from Torhorst et al. {35}

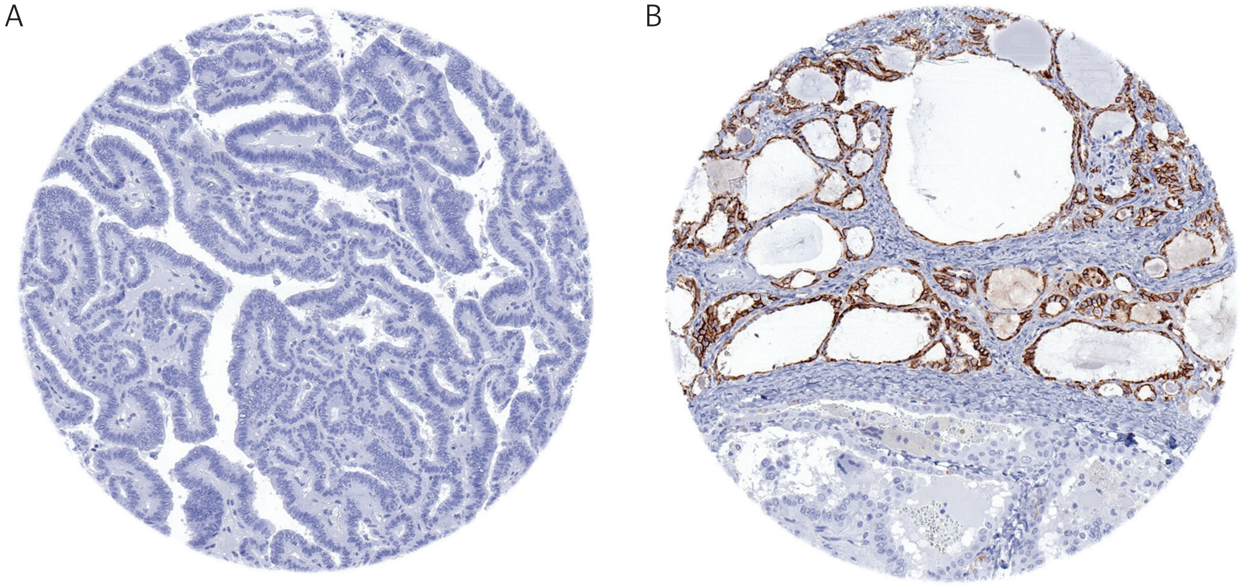

Figure 4. CDH16 deficient papillary thyroid carcinomas. **A**) CDH16 negative papillary carcinoma. **B**) CDH16 negative papillary carcinoma cells adjacent to CDH16 positive normal thyroid follicles

Figure modified from Lennartz et al. {11}.

sues and results in a complete MTAP expression loss, which can be visualised by IHC. Our comprehensive MTAP data highlighted numerous indications where MTAP deficiency can support a diagnosis of a neoplastic disease, for example pancreatic adenocarcinoma, lung cancer, melanoma, urothelial cancer, and urothelial dysplasia [15]. Our study on CDH16 confirmed that this protein is expressed in only a few cancers and can be used as a marker for renal cell carcinoma [11]. Even more interestingly and previously unknown, we also found that a loss of CDH16 is a common phenomenon in neoplastic follicular cells of the thyroid, which was seen in 93% of papillary carcinomas, 40% of follicular carcinomas, and 14% of adenomas. Examples of CDH16 deficient papillary thyroid cancers are given in Figure 4. Our study of CDH17 expression in 14,948 tumours from 150 tumour entities revealed positivity in many different tumour types but also a higher sensitivity for determining a gastrointestinal or biliopancreatic tumour origin than obtained by the alternative markers CK20 or SATB2 [16].

Characterisation of targets with gradual expression

It is a particular advantage of TMAs that studies on hundreds or thousands of tumours can be executed by using different assays such as two independent methods (i.e. fluorescence *in situ* hybridisation [FISH] vs. IHC), different antibodies, or variable antibody concentrations. The capacity of this approach was highlighted in a recent study on the diagnostic role of TRPS1 IHC [19]. RNA data from the The Cancer Genome Atlas (TCGA) [49] database suggest that TRPS1 expression might be highly specific for breast cancer. In line with this observation, initial studies on TRPS1 IHC have described high TRPS1 positivity rates in breast cancer, and because TRPS1 positivity was also frequent in triple-negative breast cancer, TRPS1 positivity was proposed as a sensitive marker for triple-negative breast cancer [50–53]. Because of emerging reports on considerable frequencies of TRPS1 positivity in tumour types other than breast cancer [53–60], we performed IHC with serial dilutions of the primary antibody on a breast cancer TMA containing > 500 tumours and a test TMA containing samples from other tumour types to identify the TRPS1 IHC protocol that resulted in an optimal specificity for the distinction of breast cancer from other tumours [19]. Using this protocol, we found TRPS1 positivity in 77% of 58 triple-negative breast cancers. Based on our observations and data from other groups describing 86–91% TRPS1 positivity in triple-negative breast cancer [52, 53], we are concerned that attempts to increase the TRPS1 staining intensity by using "optimised" protocols will have resulted in much lower specificity for breast cancer than anticipated by the users in many laboratories.

Identification of completely new targets

In an endeavour to assess antibodies against previously unexplored targets, we have tested more than 6000 different antibodies. Several of these have developed into highly useful novel diagnostic markers. Expression of carboxypeptidase A1 (CPA1), a zinc metalloprotease produced in pancreatic acinar cells, which is needed to cleave aromatic amino acids from dietary proteins, was identified to be highly specific for pancreatic acinar cell carcinoma [4]. Glutamate decarboxylase 2 (GAD2), a glutamate decarboxylase with a role in insulin-producing β-cells of pancreatic islets, was identified as a marker for pancreatic origin of neuroendocrine neoplasms [18]. Lysine demethylase 6A (KDM6A), an ubiquitously expressed nuclear protein with a role in chromatin modification and transcription control, was found to be completely lost in about 40% of low-grade urothelial carcinomas and somewhat less frequently in high-grade and invasive cancers [44, 61]. Because of its high-level expression in normal cells and its dichotomous staining pattern as either expressed at high levels or being completely absent, KDM6A staining has turned out to be a powerful tool to distinguish neoplastic cells in urine cytology, flat urothelium, and cases with equivocal morphology (Figure 5). Uroplakin 3b (UPK3B), a component of apical asymmetrical unit membrane (AUM) plaques with a pivotal role in the stabilisation and strengthening of certain cell types, was only found in three normal cell types, all of which periodically undergo extensive distension. These include surface epithelial cells of the urothelium, amnion cells, and mesothelial cells [43]. In a study on 17,693 tumours of 151 tumour entities, Upk3b expression was identified as a highly specific marker for malignant mesothelioma [43]. Uroplakins 1b (UPK1B) and 1a (UPK1A), two further AUM plaque proteins, were found to have considerable specificity for urothelial carcinoma, a tumour entity that is difficult to distinguish from other solid cancers by morphology alone [45, 46].

Vision for the future

In our institution we regularly use the data from our studies for our routine diagnostic work. Considering that it is hardly possible for individual pathologists to know the likelihood of a positive staining for each antibody in every cancer entity, we have visualised compiled data from our large-scale studies including an organ-systematic representation and a ranking order (Figure 6) on the homepage of the antibody provider (MS Validated Antibodies GmbH, Hamburg, Germany, https: //ms-validatedantibodies.com) under "Compatibility of antibodies". It is convenient to look up these data to better understand immunostaining findings on tumours of unknown origin. Our current database involves data from 61 diagnostic marker studies. Two or three markers were combined in some of these datasets because of their obvious complementarity such as TTF1 and napsin A for the distinction of pulmonary adenocarcinoma [22], TRPS1 and GATA3 for the differentiation of breast cancer [19], CPA1, CELA3B, and GP2 for the distinction of pancreatic acinar cell carcinoma [62], or CDH17, CK20, and SATB2 for the identification of a gastrointestinal or biliopancreatic tumour origin [16]. However, the degree of combinatory evaluation of data from large-scale TMA studies and their presentation could be markedly further improved. Optimally, all diagnostically useful antibodies should be evaluated on a defined set of tumours. The availabil-

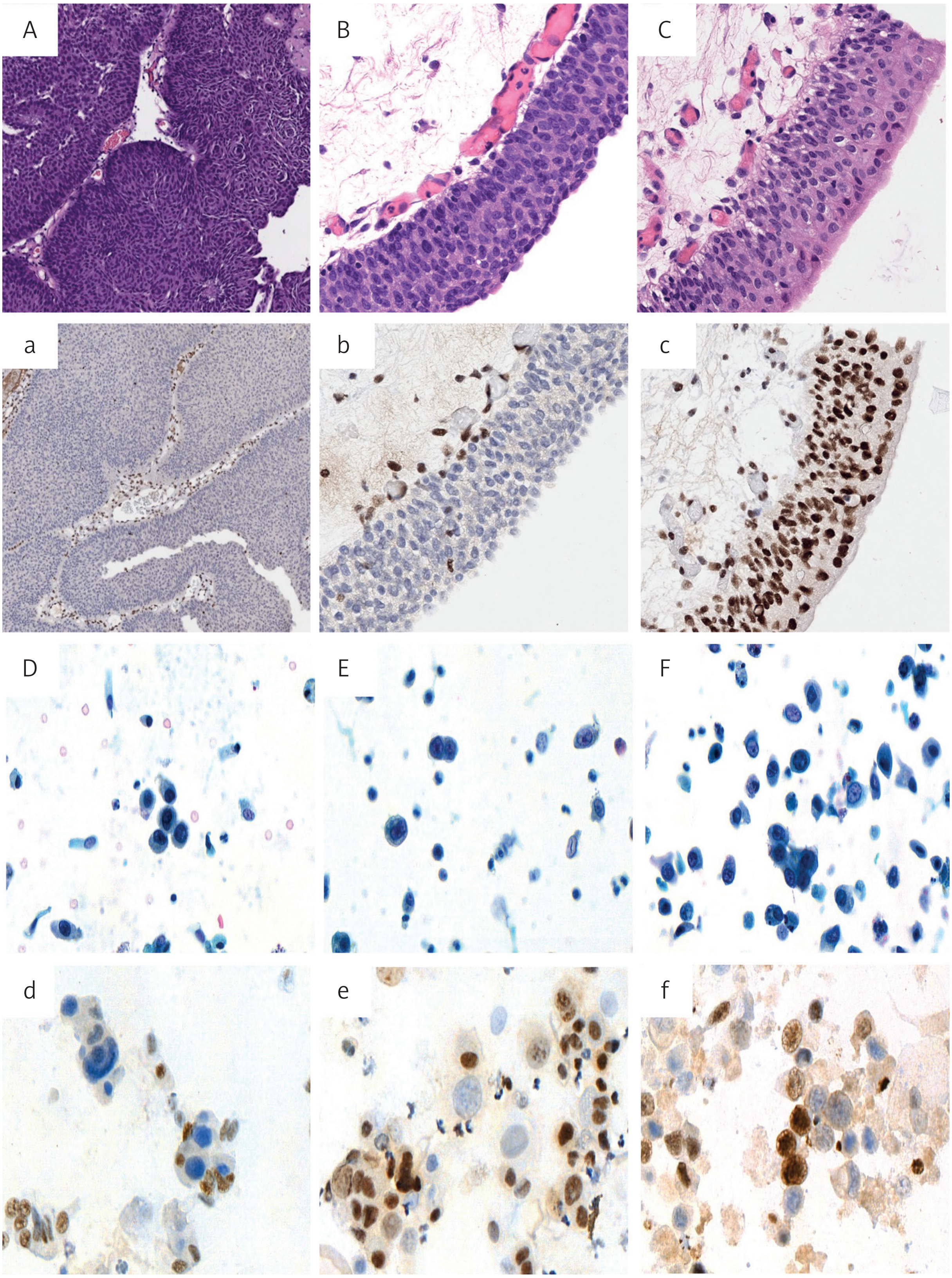

Figure 5. Lysine demethylase 6A (KDM6A) staining for distinguishing neoplastic urothelial cells. Hematoxylin and eosin staining of a urothelial carcinoma of the bladder (**A**) and normal appearing adjacent urothelium (**B, C**). Complete loss of KDM6A staining in the carcinoma (**a**) and in one adjacent normal appearing area (**b**) indicating neoplastic transformation (dysplasia), while KDM6A expression is retained in another area of tumour adjacent urothelium now considered normal (**c**). Panels **D–F** show three cases of Papanicolaou-stained urine cells with high grade urothelial carcinoma cells. KDM6A staining of these samples reveals a complete KDM6A loss in the atypical cells while normal cells are KDM6A positive (**d–f**)

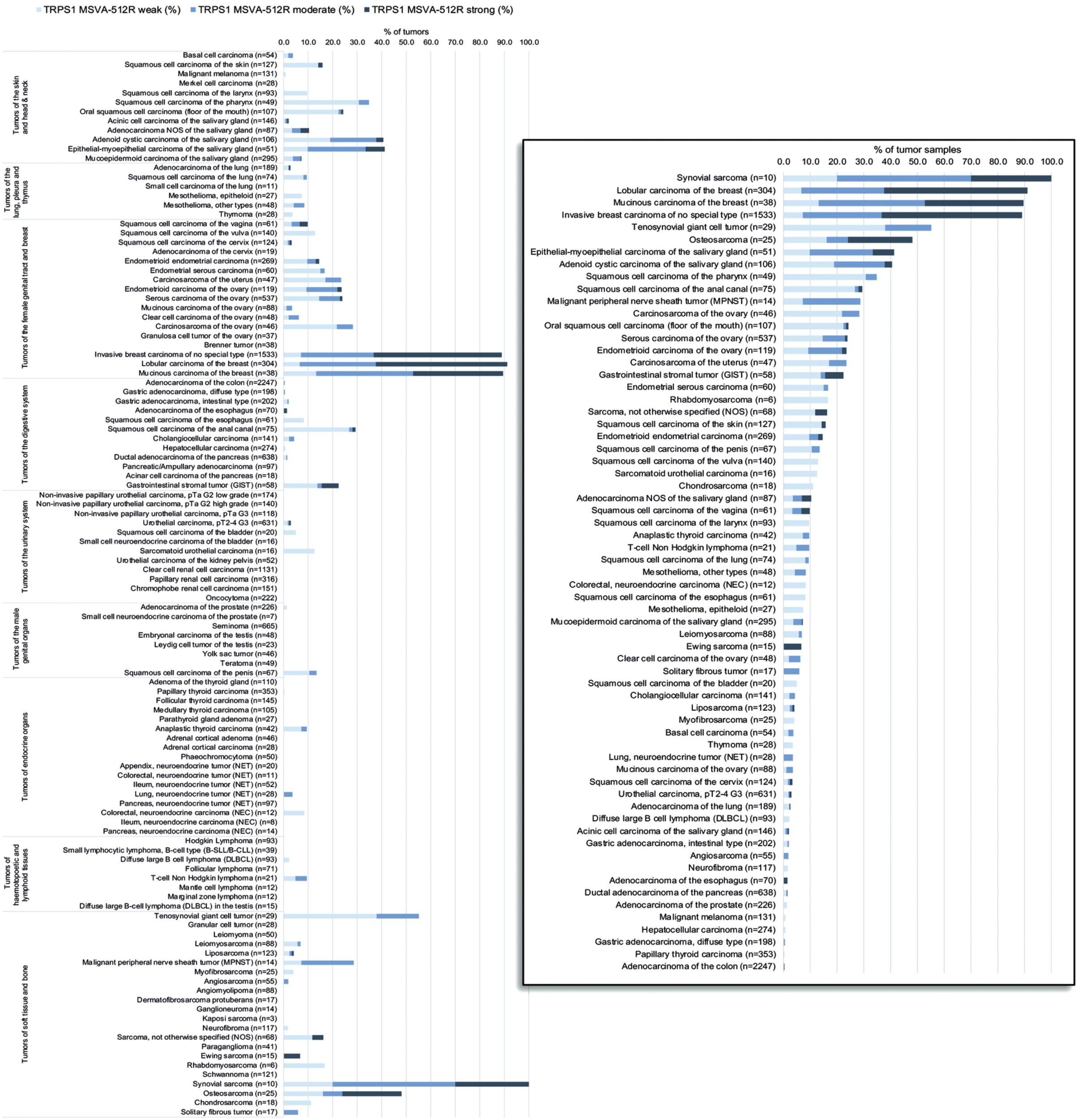

Figure 6. Visualisation of tissue microarray data compiled from our large-scale studies including an organ-systematic representation and a ranking order. The example shows data on TRPS1

Figure modified from https: //ms-validatedantibodies.com/product/trps1-msva-512r/.

ity of 6000–10,000 digitised tumour images from as many different tumour entities as possible for each of the 120-250 antibodies of a basic diagnostic portfolio would – together with HE images – represent an ideal starting resource for developing artificial intelligence (AI) tools for automated tumour categorisation. Based on HE images and clinical information, an AI could automatically select the optimal panel of antibodies, potentially suggest further analyses based on an automated evaluation of the initial IHC staining, and eventually propose a list of possible differential diagnoses together with their respective probabilities.

Disclosures

1. Institutional review board statement: Not applicable.
2. Assistance with the article: The antibodies were provided from MS Validated Antibodies GmbH, Hamburg, Germany (and ardoci GmbH, Hamburg owned by a family member of GS). Marco Sauter and Sandra Dieckert are employees of MS Validated Antibodies GmbH (MS) and ardoci GmbH (SD).
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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