Generation and evaluation of a monoclonal antibody, designated MAdL, as a new specific marker for adenocarcinomas of the lung

H Schultz¹, S Marwitz¹, B Baron-Lühr², G Zissel³, C Kugler⁴, KF Rabe⁴, P Zabel⁵, E Vollmer¹, J Gerdes² and T Goldmann*¹,¹

¹Clinical and Experimental Pathology, Research Center Borstel, Parkallee 1-40, D-23845 Borstel, Germany; ²Tumor Biology, Research Center Borstel, Parkallee 1-40, D-23845 Borstel, Germany; ³Department for Pneumology, University of Freiburg, Kilianstrasse 5, D-79106 Freiburg, Germany; ⁴Hospital Großhansdorf, Währendamm 80, D-22927 Großhansdorf, Germany and ⁵Medical Clinic, Research Center Borstel, Parkallee 1-40, D-23845 Borstel, Germany

BACKGROUND: Different therapy regimens in non-small-cell lung cancer (NSCLC) are of rising clinical importance, and therefore a clear-cut subdifferentiation is mandatory. The common immunohistochemical markers available today are well applicable for subdifferentiation, but a fraction of indistinct cases still remain, demanding upgrades of the panel by new markers.

METHODS: We report here the generation and evaluation of a new monoclonal antibody carrying the MAdL designation, which was raised against primary isolated human alveolar epithelial cells type 2.

RESULTS: Upon screening, one clone (MAdL) was identified as a marker for alveolar epithelial cell type II, alveolar macrophages and adenocarcinomas of the lung. In a large-scale study, this antibody, with an optimised staining procedure for formalin-fixed tissues, was then evaluated together with the established markers thyroid transcription factor-1, surfactant protein-A, pro-surfactant protein-B and napsin A in a series of 362 lung cancer specimens. The MAdL displays a high specificity (>99%) for adenocarcinomas of the lung, together with a sensitivity of 76.5%, and is capable of delivering independent additional diagnostic information to the established markers.

CONCLUSION: We conclude that MAdL is a new specific marker for adenocarcinomas of the lung, which helps to clarify subdifferentiation in a considerable portion of NSCLCs.

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Lung cancer is one of the leading causes of death worldwide, with a still rising incidence (Van Lerberghe et al, 2008). There are long-standing differences between the treatment regimens for the main subtypes of lung cancer, which is primarily divided into small-cell lung cancer (SCLC) and non-SCLC (NSCLC). Differentiation among SCLC, NSCLC and also metastases is of large therapeutic relevance. With regard to this, appropriate immunohistochemical procedures have been developed (Kaufmann et al, 1997).

Novel chemotherapeutic approaches have recently been developed for NSCLC, which is largely known as a chemoresistant tumour. In the respective clinical studies, substantial differences between adenocarcinomas and squamous cell carcinomas or different types of large-cell carcinomas have been shown with regard to the adequate therapeutic regimens (Smit et al, 2001; Esteban et al, 2009; Kim et al, 2009; Lee et al, 2009). Therefore, subdifferentiation of NSCLC, which to some degree has been an academic issue in the past, is currently getting more into focus as increasingly becoming a check element within therapeutic decisions.

For such subdifferentiation strategies among NSCLC, different elements of the pulmonary surfactant system repeatedly proved to be reliable markers for adenocarcinomas of the lung. These comprise members of the surfactant proteins themselves, such as surfactant protein-A (SP-A) and pro-surfactant protein-B (SP-B), which are highly specific markers but lack sensitivity (Mizutani et al, 1988; Brasch et al, 2003). Thyroid transcription factor-1 (TTF-1), a positive regulator of surfactant protein promoter activity, has emerged as a major and sensitive marker for a lung cancer, but is not up to differentiate the different entities of pulmonary cancer, such as adenocarcinoma, large-cell neuroendocrine carcinoma, small-cell carcinoma or carcinoid (Folpe et al, 1999; Barlesi et al, 2005). In addition, it designates thyroid carcinomas and their metastases (Boggaram et al, 2003; Kargi et al, 2007). The aspartic protease family member napsin A is involved in processing of SP-B in alveolar epithelial cell type II (AECII), and can be used as a marker for adenocarcinomas of the lung (Chuman et al, 1999; Hirano et al, 2003; Dejmek et al, 2007).

Despite the information obtained by the use of these established markers, there is still a need for additional diagnostic information in a portion of NSCLC.

With regard to this, we initialised a screening approach starting with immunisation of mice against human primary AECII (Zissel et al, 2007). Upon screening, one clone (MAdL) was identified as a marker for alveolar epithelial cell type II, alveolar macrophages and adenocarcinomas of the lung. In a large-scale study, this antibody, with an optimised staining procedure for formalin-fixed tissues, was then evaluated together with the established markers thyroid transcription factor-1, surfactant protein-A, pro-surfactant protein-B and napsin A in a series of 362 lung cancer specimens. The MAdL displays a high specificity (>99%) for adenocarcinomas of the lung, together with a sensitivity of 76.5%, and is capable of delivering independent additional diagnostic information to the established markers.
et al., 2000). After generation of numerous hybridomas, the corresponding monoclonal antibodies were subjected to primary screening using cell cultures and human tissues. One of the clones directed against a cytoplasmic fraction of AECII showed reactivity with AECII, alveolar macrophages and adenocarcinomas of the lungs, which was further verified using tissue microarrays (TMAs) from NSCLC tissues treated with the HOPE technique (Srinivasan et al., 2002; Goldmann et al., 2003, 2005). This clone was designated MAdL (marker for adenocarcinomas of the lung). Subsequently, an optimised protocol for the use of MAdL on formalin-fixed, paraffin-embedded (FFPE) tissues was developed. Here, we present the results of the use of MAdL in addition to the above-mentioned established markers.

**MATERIALS AND METHODS**

**Primary human AECII**

Samples from macroscopically tumour-free lung tissue were cut from the surgical specimens and used for cell isolation procedure as described previously (Pechkovsky et al., 2005, 2006). In brief, the lung tissue was first sliced and slices were washed three times at 4 °C in PBS. The washed slices were incubated in sterile dispase solution at 37 °C for 45 min. After dispase digestion, the lung tissue slices were cut into small, pipettable pieces and thoroughly pipetted for several minutes. Crude tissue and cell suspensions were filtered through nylon gauze with meshes of 100 μm, 50 μm and 20 μm. The resulting single-cell suspension was placed on Ficoll separating solution and centrifuged at 800 g for 20 min. The AECII-enriched cells from the interface were washed and incubated in 100 mm plastic dishes at 37 °C in humidified air containing 5% CO₂ for 15, 20 and 30 min, with seeding of non-adherent cells on fresh dishes for each time interval to remove adherent cells (alveolar macrophages, monocytes, fibroblasts and endothelial cells). To remove remaining monocytes/macrophages and lymphocytes, antibodies against CD3 (OKT3 and ECACC 86022706) and CD14 (HB-246 ATCC) were added and the antibody-binding cells were removed by anti-mouse IgG-coated magnetic beads and magnetic antibodies against CD3 and CD14 (Immunotech, Marseille, France) as previously described (20). Viability of the AECII after isolation was >97%, as determined by trypan blue exclusion. After the final step of MACS purification, the AECII preparations included in this report were free of CD14⁺ and CD3⁺ cells, as determined by immunocytochemistry. In all, 98 ± 1.3% of cells were identified as AECII by the presence of dark blue inclusions, as revealed by immunocytochemistry. In all, 98 ± 1.3% of cells were identified as AECII by the presence of dark blue inclusions, as revealed by immunocytochemistry. In all, 98 ± 1.3% of cells were identified as AECII by the presence of dark blue inclusions, as revealed by immunocytochemistry. In all, 98 ± 1.3% of cells were identified as AECII by the presence of dark blue inclusions, as revealed by immunocytochemistry.

**Generation of the monoclonal antibody**

A total of 1 × 10⁷ cells were thawed and washed twice in 0.9% NaCl solution. Subsequently, the cells were lysed in 1 ml lysis solution (0.9% NaCl and 0.5% Tween 20) and vortexed for 1 min. The lysate was cleared by centrifugation at 34 g for 15 min and the supernatant stored at −20 °C. A female Balb C mouse was immunised by subcutaneous injections using a mixture of cell lysate and GERBU Adjuvant MM (Gerbu Biotechnik, Gaiberg, Germany): day 0, 60/40 μl; days 14 and 21, 30/20 μl; and days 28, 29 and 30, 50 μl lysate only. To generate antibody-producing hybridoma lines, spleen cells of this mouse were fused on day 31 with × 63 Ag8.6.5.3 myeloma cells using a standard PEG-based procedure (Köhler and Milstein, 1975). Antibody clones were assessed for their staining pattern using cytospins of primary AECII cells as well as sections of normal human lung and tonsil.

**Immunohistochemistry**

For a preliminary study of the immune reactivity of MAdL, TMAs were produced from 35 HOPE-fixed, paraffin-embedded NSCLC tissues as previously described (Goldmann et al., 2005). HOPE-fixed specimens allow immunohistochemistry (IHC) without antigen retrieval (AR), which could create artefacts in staining or morphology (Goldmann et al., 2003).

For immunodetection of MAdL, 1 μm thick sections of HOPE-fixed, paraffin-embedded tissues were deparaffinised by two times of incubation in isopropanol for 10 min at 65 °C. Deparaffinised sample slides were air dried at room temperature and rehydrated for 10 min in 70% (v/v) aceton/DEPC-treated water at 4 °C. Remaining acetone was removed by incubation for 10 min in DEPC-treated water at 4 °C and transferred into distilled water at room temperature. Endogenous peroxidases were blocked for 10 min in 3% H₂O₂ solution. A volume of 2 μg ml⁻¹ of isolated MAdL was diluted with antibody diluent (Zytomed Systems, Berlin, Germany) and applied for 60 min in a moist chamber. For blocking and detection, a HRP-conjugated polymer kit according to the manufacturer’s instructions (Zytomed Systems) was used. Washing steps were carried out three times for 5 min after each reagent step with washing buffer (50 μm Tris saline buffer with 0.1% (v/v) Tween 20; pH 7.6). Negative controls were included under omission of secondary antibody. Permanent AEC (permanent AEC Kit, Zytomed Systems) was used as substrate for HRP-conjugated polymer. Colour reaction was stopped with distilled water. Samples were dehydrated in increasing concentrations of ethanol, washed for 20 s in xylene and cover slips were mounted using Pertex (Medite, Burgdorf, Germany) as mounting medium.

**IHC with MAdL on FFPE tissues**

As most tissue specimens are classically fixed with 4% neutral-buffered formalin in routine diagnostics, IHC has usually faced the cross-linking effects (Srinivasan et al., 2002) before conducting immunodetection. To increase the diagnostic value, MAdL had therefore to be applicable on FFPE specimens. For an optimal staining result on FFPE specimens, heat-induced AR was tested with acidic, as well as alkaline buffers and standard enzymatic digest (see Table 3). Finally, enzyme-based pretreatment with Fast Enzyme (Zytomed Systems) for 3 min at ambient temperature or Proteinase XXV (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at ambient temperature turned out to provide the best results.

Immunohistochemistry with FFPE tissues was generally conducted as mentioned above, with the exception that FFPE-tissue slides were deparaffinised by incubation in xylene (2 × 10 min) and subsequent rehydration in a graded ethanol series for 2 min each step (2 × 100, 2 × 96, 90, 80, 70%, 2 × distilled water). The primary antibodies were applied for 1 h at ambient temperature as described in Table 2. Negative controls were included under omission of primary antibody in each staining series, as well as positive reference sections from human lung to ensure even results.

**Screening for cross-reactivity in malignant and non-malignant human tissues**

For evaluation of affinity and cross-reactivity in human tissues, the antibody was tested on various malignant pulmonary (see Table 3) and non-malignant tissues (see Table 4) from either the lung or the other functional systems. In short, the expression of MAdL, in addition to its expression in human lung adenocarcinomas, was...
investigated in the most common lung-metastasising tumours. To exclude false-positive reactions with other non-malignant tissues, MADL was analysed for expression in any functional system, including digestive and urogenital tract, as well as connective tissue, nervous system, endocrine organs and the skin.

Study group

Patient materials were obtained from either lobectomy, pneumonectomy or peribronchial biopsy at the Hospital of Großhansdorf or Medical Clinic Borstel, Germany. All used archived FFPE tissue blocks were of pathologically and clinically proven diagnosis.

The expression of MADL was evaluated and compared with common markers for adenocarcinomas of the lung in 362 primary lung carcinomas. The group consisted of 154 squamous cell carcinomas, 167 adenocarcinomas, 2 adenosquamous carcinomas, 19 small-cell carcinomas, 17 large-cell carcinomas and 3 carcinoids. From each diagnosis, an almost comparable amount of specimens was used from either surgical or biopsy origin, in order to compare possible expression differences (260 surgical specimens and 201 biopsy specimens). In addition to primary lung carcinomas, expression of MADL was investigated in 111 non-pulmonary carcinomas. This series comprised of 28 colon carcinomas, 19 mamma carcinomas, 11 prostate carcinomas, 6 pancreas carcinomas, 10 gastric carcinomas, 21 kidney

| Tumour entity | Grading (N) | Surgical | Biopsy | TTF-1 | MADL | SP-A | SP-B | CK 5/6 |
|---------------|------------|----------|--------|-------|------|------|------|--------|
| Primary lung carcinomas | | | | | | | | |
| Squamous cell carcinoma (154, 122/32, 66.7) | G1 (9), G2 (63) | 77 | 77 | 0 | 0 | 0 | 0 | 154 (100) |
| Adenocarcinoma (167, 84/83, 63) | G1 (4), G2 (77), G3 (87) | 87 | 78 | 154 (92.2) | 124 (74.2) | 92 (55) | 88 (52.6) | 0 |
| Small-cell carcinoma (19, 109, 66.3) | G2 (19) | 4 | 5 | 14 (73.6) | 0 | 0 | 0 | 0 |
| Large-cell carcinoma (17, 161, 62.7) | G3 (17) | 4 | 13 | 4 (23.5) | 0 | 0 | 0 | 0 |
| Adenosquamous carcinoma (2, 111, 61.5) | G2 (2) | 2 | 0 | 2 (100) | 2 (100) | 1 (50) | 1 (50) | 2 (100) |
| Carcinoids (3, 1/2, 68.7) | G2 (3) | 0 | 3 | 2 (66.6) | 0 | 0 | 0 | 0 |
| Other carcinomas | | | | | | | | |
| Colon carcinoma (28, 711/1, 72.3) | G2 (18), G3 (10) | 24 | 4 | 0 | 0 |
| Mamma carcinoma (19, 0/19, 60.5) | G1 (1), G2 (7), G3 (11) | 14 | 5 | 0 | 0 |
| Mesothelioma (8, 71, 69.7) | G2 (1), G3 (7) | 0 | 8 | 0 | 0 |
| Prostate carcinoma (11, 110, 64) | G2 (5), G3 (6) | 11 | 0 | 0 | 0 |
| Pancreas carcinoma (6, 3/3, 63.6) | G2 (1), G3 (5) | 6 | 0 | 0 | 0 |
| Gastric carcinoma (10, 91, 78.6) | G2 (4), G3 (6) | 4 | 6 | 0* | 0 |
| Renal carcinoma (21, 111, 60) | G1 (2), G2 (9), G3 (10) | 21 | 0 | 1 (4.7)b | 0 |
| Bile duct carcinoma (1, 1/0, 79) | G3 (1) | 0 | 1 | 0 | 0 |
| Hepatocellular carcinoma (1, 10, 45) | G2 (1) | 1 | 0 | 0 | 0 |
| Endometrium carcinoma (3, 0/3, 68) | G2 (2), G3 (1) | 3 | 0 | 0 | 0 |
| Thyroid carcinoma (1, 0/1, 64) | G2 (1) | 1 | 0 | 0 | 0 |
| Urothelium carcinoma (2, 2/0, 70) | G2 (1), G3 (1) | 1 | 1 | 0 | 0 |

Abbreviations: CK = cytokeratin; f = female; m = male; SP = surfactant protein; TTF = thyroid transcription factor. *Not relevant for diagnostics. bChromophile renal cell carcinoma.

Table 3 Investigated specimens

Table 2 Applied antibodies and AR for immunohistochemistry

Table 1 Assessment of AR for MADL on FFPE specimens

Abbreviations: AR = antigen retrieval; FFPE = formalin fixed, paraffin embedded. Result: – = unsufficient staining quality; + = week staining quality; ++ = moderate staining quality; +++ = good staining quality.
RESULTS

Establishing of IHC and screening of non-malignant tissues

In a prescreening study, HOPE-fixed carcinomas of the lung were investigated by application of MAdL culture supernatants without any AR. In all, 80% of investigated adenocarcinomas (16 out of 20) were positive and displayed a cytoplasmic, granular signal in epithelia of the tumour. All tested squamous cell carcinomas (20) were negative for MAdL (data not shown).

Results of the prescreening study were verified on formalin-fixed adenocarcinomas under equal conditions after establishing optimal AR conditions. A broad range of commonly used AR methods has been applied and compared to optimise the MAdL staining protocol with FFPE tissues. No heat-induced AR, with both acidic and alkaline buffer, resulted in reasonable staining. The same holds true for enzymatic digests with proteinase K, both acidic and alkaline buffer, resulted in reasonable staining.

The staining was found in lymphoid tissue (Figure 1E and F).

Comparison of MAdL expression with common markers for adenocarcinomas of the lung

For investigation of MAdL expression and comparison with common applied markers for diagnosis of adenocarcinomas of the lung, a cohort of lung carcinomas was screened. All specimens were analysed for expression of MAdL, TTF-1, SP-A, SP-B and, in case of squamous cell carcinoma, cytokeratin 5/6.

Results for all the markers are displayed in Table 3, including histomorphological entities and expression profiles. In case of adenocarcinomas, 90.2% showed positive expression of TTF-1 and 74.2% were positive for MAdL. Staining targeting the surfactant proteins SP-A and SP-B revealed 55% and 52.6% positivity, respectively (Figure 2A). No expression of TTF-1, MAdL, SP-A and SP-B could be observed in squamous cell carcinomas, whereas all of them displayed a positive signal for cytokeratin 5/6. Neither small-cell and large-cell carcinomas, nor carcinoids were positive for MAdL or surfactant proteins. On the contrary, TTF-1 expression was observed in 73.6% of small-cell carcinomas, in 23.5% of large-cell carcinomas and in 66.6% of atypical carcinoids.

The two investigated adenosquamous lung tumours were all positive for TTF-1 and MAdL in adeno-differentiated part and CK 5/6 in squamous-differentiated part, respectively. Only one case was positive for both SP-A and SP-B. Within the group of non-pulmonary tumours, MAdL immunoreactivity could only be observed in one case of chromophile renal cell carcinoma (Figure 1D). Other investigated subtypes of renal carcinoma such as papillary and clear-cell renal cell carcinoma showed no expression.

Marker expression depending on tumour grading and specimen origin

Regarding the grading of investigated adenocarcinomas, only a small number (four) were low-grade (G1) carcinomas. The majority (77 cases) consisted of intermediate- (G2) and 87 cases of high-grade carcinomas (G3). In routine diagnostics, the intermediate- and high-grade adenocarcinomas of the lung are far more frequent. Therefore, it is helpful to have a marker at hand, which maintains its expression qualitatively even in poorly differentiated carcinomas. With respect to TTF-1, its expression did not reduce dramatically in intermediate- and high-grade adenocarcinomas. The novel aspartic proteinase napsin A showed no expression in one case of chromophile renal cell carcinoma (Figure 1D). Other investigated subtypes of renal carcinoma such as papillary and clear-cell renal cell carcinoma showed no expression.

Table 4 Expression of MAdL in non-malignant tissues

| Investigated tissues                  | No expression | Expression |
|---------------------------------------|---------------|------------|
| Respiratory system                    |               |            |
| Respiratory epithelium                |               | Pneumocyte type II |
| Peribronchial glands                  |               | Alveolar macrophages |
| Pneumocyte type I                     |               |            |
| Digestive tract                       |               |            |
| Gastric mucosa                        |               |            |
| Duodenum mucosa                       |               |            |
| Small-intestine mucosa                |               |            |
| Colon mucosa                         |               |            |
| Liver parenchyma                      |               |            |
| Bile duct and bladder                 |               |            |
| Pancreas parenchyma                   |               |            |
| Urogenital tract                      |               |            |
| Kidney                                | Tubules, glomeruli | Proximal tubules |
| Efferent urinary system               | Urothelia     |            |
| Prostate                              | Seminal vesicle|            |
| Testis                                | Seminal epithelium |            |
| Connective tissue                     | Smooth/skeletal muscles |            |
| Heart muscle                          | Adipocytes    |            |
| Adipocytes                            | Fibroblasts   |            |
| Nervous system                        | Nerve (autonomous and somatic) |            |
| Ganglions                             | Brain         |            |
| Endocrine organs                      | Adrenal gland |            |
| Pituitary gland                       | Thyroid gland |            |
| Parathyroid gland                     | Langerhans islet cells |         |
| Skin                                  | Epidermis     |            |
| Melanocytes                           | Integumentary appendage |          |

carcinomas, 1 bile duct carcinoma, 1 hepatocellular carcinoma, 1 thyroid carcinoma, 3 endometrium carcinomas and 2 urothelium carcinomas, as well as 8 cases of epitheloid mesothelioma. Detailed diagnostic information for each diagnosis is summarised in Table 3.

Diagnosis and grading of investigated specimens was conducted according to the WHO Classification of Tumours 2004.
As a growing number of samples results from diagnostic biopsies, we intended to analyse and compare the sensitivity of MAdL and other markers between specimens of surgical and biopsy origin. Regarding TTF-1, there was a high sensitivity (47.5%) in both surgical and biopsy specimens. Interestingly, it could be observed that in surgical specimens, the sensitivity of MAdL was even a little bit higher compared with TTF-1. The surfactant proteins SP-A and SP-B showed a moderate expression in surgical tissues (57% and 56%, respectively). Emphasising on biopsy tissues, MAdL displayed moderate sensitivity (56%) in contrast to SP-A and SP-B, which declined to 44.9 and 41%, respectively (Figure 2C).

Expression patterns of TTF-1, MAdL, SP-A and SP-B in adenocarcinomas of the lung

Among the investigated 167 cases of adenocarcinomas, the majority was positive for all the applied markers (32.7%). Thyroid transcription factor-1 expression alone was observed in 21 (12.5%) and MAdL alone in 2 (1.2%) cases. Within the investigated group, the expression of both TTF-1 and MAdL comprised about 25 cases (14.9%) and was the second most prevalent observed pattern. A combination of either TTF-1, MAdL and SP-B (9.5%), or TTF-1, MAdL and SP-A (12.5%) was of third most incidence (Figure 3).

MAdL in comparison with TTF-1, SP-A and SP-B

In addition to routine cases in diagnostics, there are repeating situations with aberrant histological specimen. As depicted in Figure 4, a pleura carcinosis from a pulmonary adenocarcinoma was negative for both SP-A and SP-B. Addressing TTF-1 could only confirm pulmonary origin of the carcinoma, although analysis of MAdL expression revealed the specific subtype. The same holds true for mixed tumour entities, which might have a squamous- as well as an adenoid-differentiated component (Figure 5). Cytokeratins 5 and 6 detect the squamous component in the left part of the photomicrograph, and TTF-1 is found to be expressed in a subgroup of glands. In contrast to SP-B, which only provides a patchy and weak signal, MAdL is expressed in the majority of adenoid glands.

DISCUSSION

Establishing MAdL staining protocol

The requirements of antibodies as markers in daily routine diagnostics have to face the molecular characteristics of the specimen analysed. As most tissues are traditionally FFPE, an AR is indispensable because of formalin’s cross-linking drawbacks (Srinivasan et al, 2002). New markers have to meet these circumstances in order to become widely applicable. As it is
hypothesised that most adenocarcinomas probably arise from bronchoalveolar stem cells, from which Clara cells or AECII derive. We immunised mice with a cytoplasmic fraction of AECII to generate a diagnostically relevant antibody to differentiate adenocarcinomas of the lung. One of the clones, designated MAdL, demonstrated a high specificity for pulmonary adenocarcinomas in HOPE-fixed tissues. Additionally, MAdL reacts with AECII and alveolar macrophages, whereas macrophages from other locations showed no reaction (Figure 1E and F). This may suggest phagocytosis rather than synthesis of the MAdL antigen in macrophages. To expand the application spectrum to bronchoalveolar stem cells, from which Clara cells or AECII derive we hypothesised that most adenocarcinomas probably arise from bronchoalveolar stem cells, from which Clara cells or AECII derive. We immunised mice with a cytoplasmic fraction of AECII to generate a diagnostically relevant antibody to differentiate adenocarcinomas of the lung. One of the clones, designated MAdL, demonstrated a high specificity for pulmonary adenocarcinomas in HOPE-fixed tissues. Additionally, MAdL reacts with AECII and alveolar macrophages, whereas macrophages from other locations showed no reaction (Figure 1E and F). This may suggest phagocytosis rather than synthesis of the MAdL antigen in macrophages. To expand the application spectrum to bronchoalveolar stem cells, from which Clara cells or AECII derive we hypothesised that most adenocarcinomas probably arise from bronchoalveolar stem cells, from which Clara cells or AECII derive.
TTF-1 can also be found in other lung tumour entities such as SCLC, large-cell neuroendocrine carcinomas and carcinoids (Folpe et al., 1999). Additionally, TTF-1 is reported to be expressed inversely in relation to tumour differentiation (Lau et al., 2002; Jagirdar, 2008; Yang and Nonaka, 2010). Regarding the reactivity with other (Klingen et al., 2010) and lung malignancies, TTF-1 is not sufficient as a stand-alone marker for diagnosis of primary lung adenocarcinomas. In addition, SP-B and SP-A are usually applied to further discriminate between squamous cell carcinomas and adenocarcinomas. For SP-A, the sensitivity ranges between 45 and 64%, and its expression declines notably with loss of differentiation (Yang and Nonaka, 2010). As the highly specific SP-A clone PE-10 (Dempo et al., 1987; Sugiyama et al., 1992; Goldmann et al., 2009) is no more commercially available and the

Figure 4 Photomicrograph of IHC on a case of pleura carcinosis from pulmonary adenocarcinoma origin. Hematoxylin–eosin-stained overview (A). No expression of either SP-A or SP-B was observed (data only shown for SP-A, B). Targeting TTF-1 resulted in strong nuclear (C) or cytoplasmic staining for MAdL (D). All images were at × 400 magnification.

Figure 5 Photomicrograph of IHC on a case of adenosquamous carcinoma of the lung. Squamous-differentiated tumour component revealed a strong CK5/6 positivity (A). Adenoid component of the tumour shows a distinct nuclear signal for TTF-1 (B) and a patchy staining for SP-B (C). Cytoplasmic signals for MAdL could be observed, in contrast to TTF-1, in the majority of glands (D).
follow-up clone displays cross-reactivity with intestinal epithelia and carcinomas (authors own observations), the specificity for pulmonary adenocarcinomas is not warranted. Therefore, for means of differentiation, SP-B can be applied but with a lack of sensitivity (52.6%, see Figure 2A). Applying MAdL as a second-line marker improves sensitivity to 74.2% in contrast to the commonly used surfactant proteins. Hence, we were able to show in an extensive study that MAdL is only expressed in alveolar macrophages, AECII and proximal tubules of the kidney in non-malignant tissues. Emphasising on malignant tissues, MAdL can exclusively be found in adenocarcinomas, whereas adenoid-differentiated extrapulmonary malignancies, which usually metastasise to the lung (Dail and Hammar, 2008), were not detected. Furthermore, only adenoid parts of adenosquamous carcinomas, as well as one case of chromophobe renal cell carcinoma, displayed positivity for MAdL. This reactivity may come in line with the observed expression in its non-malignant tissue origin, the proximal renal tubules (Figure 1A). In contrast to TTF-1, no reactivity with non-adenous lung carcinomas, such as neuroendocrine carcinomas, large-cell neuroendocrine carcinomas, as well as small-cell bronchial carcinoma and carcinoids, was observed. This counts for a superior quality of MAdL as a second-line marker for subdifferentiation of lung cancer. Owing to the way of generating MAdL by immunisation of mice with preparations of fractions of primary human AECII, we do not yet know the target molecule of MAdL. The knowledge of this target molecule would allow insights into the biological relevance of MAdL expression in different tissues. Appropriate studies are currently underway to uncover this target molecule, which use techniques such as two-dimensional gel electrophoresis, immunoprecipitation techniques, accompanied by mass spectrometry and immunogold electron microscopy.

Sensitivity of MAdL persists in biopsy material and during dedifferentiation

Biopsy specimens, in general, inhabit only a small fraction of the whole tumour compared with surgical specimens. Therefore, the heterogeneity in expression of applied markers has a more severe effect on the sensitivity. In contrast to SP-A (44.9%) and SP-B (41%), MAdL retained a higher sensitivity (56.4%) in biopsy material. Interestingly, MAdL (88.8%) showed a comparable sensitivity to TTF-1 (85.7%) in surgically resected specimens (Figure 2C). In addition to sensitivity in biopsy specimen, we further investigated the sensitivity of MAdL depending on tumour grading. In general, there is up to date no universal grading system for adenocarcinomas of the lung that is not disputed (Travis et al., 2011). However, it is widely accepted that loss of differentiation by increasing grading reflects likelihood of lymph node metastasis and reoccurrence after surgical intervention (Chung et al., 1982). Thyroid transcription factor-1 and napsin A both show an impressing sensitivity almost independent of grading, but with a noteworthy lack of specificity for adenocarcinomas of the lung. The surfactant proteins range lower in sensitivity and suffer, in addition, from a loss in G2 to G3 tumours. The MAdL as a marker with sensitivity in between napsin A and SP-A or SP-B will add a decent advantage based on its specificity and persistent expression in terms of grading stages or specimen origin.

Diagnostic benefit of applying MAdL

The currently arising targeted therapies comprising receptor tyrosine kinase inhibitors such as gefitinib or erlotinib offer a true benefit for the patients (Mok et al., 2009). With regard to the therapy decision, one has to bear in mind that this is not exclusively achieved by molecular-based analysis as for EGFR mutations (Rosell et al., 2009), but also deeply depends upon the previous diagnosis of NSCLC and subdifferentiation into adenocarcinomas. Without a concrete and specific diagnosis, the subsequent molecular-based analyses and therapies will be impaired. Here, MAdL as a new and specific marker for adenocarcinomas of the lung offers a diagnostic benefit of 16% if used as a second-line marker besides TTF-1. As 12.7 × 106 cases of cancer have been globally diagnosed in 2008, with 13% (1.6 × 106) of which are lung cancers (Jemal et al., 2011), with almost 50% of adenocarcinomas (Curado et al., 2007), a 16% higher diagnosis because of MAdL would account for 132,000 more cases each year worldwide. In addition to EGFR-targeting therapies, new molecular insights into lung cancer development currently have emerged. K-RAS mutations appear in 25% of adenocarcinomas and may be a promising target of new therapeutic strategies (Sunaga et al., 2011), as well as the recently described EML4-ALK oncoprotein counterpart (Gerber and Minna, 2010). This growing number of insights into molecular events during lung cancer development will doubtless lead to better therapy, but the diagnosis of certain NSCLC subtypes has to keep pace with these events. Therefore, we developed MAdL as a new second-line marker for adenocarcinomas of the lung that might improve the primary diagnoses on which targeted therapies depend.

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