Determination of PD-L1 Expression in Effusions From Mesothelioma by Immuno-Cytochemical Staining

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BACKGROUND: Malignant mesothelioma (MM) is an aggressive, fatal tumor. Current therapeutic options only marginally improve survival. Programmed cell death ligand 1 (PD-L1) is a dominant mediator of immunosuppression, binding to programmed cell death 1 (PD-1). PD-L1 is up-regulated in cancer cells, and the PD-1/PD-L1 pathway plays a critical role in tumor immune evasion, thus providing a target for antitumor therapy. Further, a correlation between PD-L1 expression and prognosis has been reported. Studies performed on histological material have revealed expression of PD-L1 in MM, but no study has been performed on MM effusions thus far.

METHODS: PD-L1 expression was determined by a commercially available antibody (clone 28-8) in 74 formalin-fixed, paraffin-embedded cell blocks from body effusions obtained at diagnosis from patients with MM. The presence of MM cells was confirmed with CK5/6, calretinin, and EMA and the admixture of macrophages was assessed with CD68. Only cases containing more than 100 tumor cells were assessed. Membranous staining in tumor cells was considered positive. Survival time was calculated from the appearance of the first malignant effusion until death.

RESULTS: Reactivity was observed in 23 of 61 (38%) of cases and was classified as ≥1%-5% (n = 9 cases), >5%-10% (n = 4 cases), >10%-50% (n = 4 cases), and >50% (n = 6 cases) positive cells. Survival times did not differ significantly between patients with PD-L1-positive and PD-L1-negative tumors.

CONCLUSION: MM effusions are suitable for immune-cytochemical assessment of PD-L1 expression in malignant cells and the results are similar to those reported for histological specimens.

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KEY WORDS: malignant mesothelioma (MM); programmed cell death ligand 1 (PD-L1); cytology; effusion; immuncytochemistry (ICC).

INTRODUCTION

Malignant mesothelioma (MM) is a highly aggressive malignant tumor arising from mesothelial cells lining the serous cavities of the body. MM originates most commonly in the pleura, to a lesser extent in the peritoneum and rarely in the pericardium. Long-term exposure to asbestos is the leading cause of mesothelioma, but in spite of the ban on asbestos in many countries, the incidence of mesothelioma continues to increase worldwide. The prognosis is poor, and survival has not improved since the 1970’s, with the median survival time remaining at 6 months. Radical surgery is only applicable to the few tumors diagnosed at an early stage and only prolongs median survival time to 12 months and the achievement of platinum/antifolate treatment is similar. Thus, the development of novel therapeutic approaches is urgently required.
There is mounting evidence that the body’s immune system is instrumental in the genesis and development of the disease, thus new therapy approaches addressing immunological mechanisms are being developed. One of the targets is the PD-1 receptor and its ligands. Programmed cell death-1 (PD-1) is a transmembrane glycoprotein with ligand specificity that is expressed in activated T lymphocytes. PD-1 is a negative regulatory receptor on T cells that plays an important role in the regulation of T cell activity and acts as a coinhibitory receptor to prevent immune activation. Programmed cell death 1 ligand 1 (PD-L1), also known as B7 homolog 1 (B7-H1), is a dominant mediator of immunosuppression and acts as a negative costimulatory molecule. When PD-L1 binds to the PD-1 receptor, the proliferation and function of activated T lymphocytes is inhibited, leading to a T cell hyporeactive condition. PD-L1 is expressed in highly variable degree on various types of cells and tissues including placenta, pancreatic islet cells, mesenchymal stem cells, some nonhematopoietic tissues, and immune cells such as macrophages and myeloid dendritic cells. PD-L1 can be upregulated in cancer cells and constitutes a mechanism for the cell to evade the immune system, avoid T cell cytosis, and facilitate cancer formation and has been shown to be widely expressed in several malignancies, including carcinoma of lung, esophagus, pancreas, ovary, breast, kidney, head and neck, and brain, among others.

Several humanized antibodies directed against PD-1 or PD-L1 are currently being tested against a range of tumors in phase 3 clinical studies, and some are already in clinical use. On theoretical grounds, it is generally assumed that the immunoassay of the expression of PD-L1 could predict the therapeutic response, and patient inclusion is based on PD-L1 positivity, though the criteria differ, from ≥1% to ≥50% of tumor cells. In a phase 1b clinical study, 25 MM patients selected for PD-L1 positivity at a 1% cutoff in tumor cell or stroma were treated with a PD-1 antibody with encouraging results. Few studies have investigated the expression of PD-L1 in MM, and of those studies, all have been performed on histological material.

Previous recommendations state that the diagnosis of MM always requires an adequate biopsy. However, according to a recent article sanctioned by the International Mesothelioma Interest Group and the International Academy of Cytology, a reliable MM diagnosis can be established in effusion cytology, although the sensitivity may be lower. Cytology is less traumatic to the patient and may lead to a diagnosis at an earlier stage, given that effusion is often the first clinical sign of MM.

The differences between effusion cytology and histology affect the criteria of assessment. The cells in effusions exist in a different environment, which may have an effect on morphological features as well as the expression of various biomarkers. Furthermore, effusions usually contain a diverse range of inflammatory cells, and the yield of malignant cells may be scanty.

Therefore, we tested the expression of PD-L1 in malignant effusions from MM cases that were histologically confirmed to investigate whether effusions are suitable for immuno-cytochemical detection of PD-L1 reactivity and whether the results are comparable with those obtained in previous studies performed on histological material. Furthermore, we correlated the expression to survival time.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded (FFPE) cell pellets from a total of 74 malignant effusions, collected from 1999 through 2016 (pleural, n = 69; peritoneal, n = 4; pericardial, n = 1) from patients with MM and diagnosed at the Department of Pathology, Skåne University Hospital, Malmö, Sweden (n = 48 cases) or the Department of Pathology and Cytology, Halland Hospital, Halmstad, Sweden (n = 26 cases) were retrieved from the archives. All samples were collected before the patients received therapy, and there were no differences in the sampling method.

Cytological diagnoses were based on wet-fixed hematoxylin and eosin stain (H&E) and air-dried May-Grünwald Giemsa stained smears (as well as FFPE cell pellets at Halland Hospital) supported by immunocytochemical staining (as well as hyaluronan analysis at Skåne University Hospital). The diagnostic immunostains were performed on FFPE cell pellets (Halland Hospital) or cytopsins (Skåne University Hospital). The immunopanels varied slightly between Halland Hospital and Skåne University Hospital and over time. At Skåne University Hospital, the basic panel consisted of CEA, EMA, and BerEp4, with the addition of CK5 and desmin in some of the later cases. At Halland Hospital, basically the same antibodies were used, although calretinin and CK5/6 were used instead of CK5 to prove the
mesothelial nature of the cells. The Halland Hospital cases were supplemented with CEA, EMA, and/or BerEp4 if any of these antibodies had not been used in the original diagnostic panel. In both hospitals, appropriate antibodies were added depending on possible differential diagnoses.

The final diagnoses were based on histological specimens, when available (n = 59 cases), in the remaining cases on all available clinical data and the results of imaging techniques. In no cases were there suspicions of another primary tumor. Patient characteristics—including age, sex, sampling date, deceased date, antibodies expression and histology subtype—were retrieved from the databases of the Department of Pathology at Skåne University Hospital and the Department of Pathology and Cytology at Halland Hospital and from the patients’ charts. Therapy could be ascertained in 42 of the cases. Twenty-three patients had received active therapy (chemotherapy [n = 17 cases]) or pleuropulmectomy, preceded by chemotherapy [n = 6 cases]). Nineteen patients had received no treatment or had been treated with pleurodesis only.

From all cell blocks, an H&E-stained slide was made and examined by two of the authors (A.D., M.S.I.M.) to confirm the presence of malignant cells. If no clearly malignant cells could be identified, the case was not included. If the material contained mesothelial cells without obvious malignant characteristics, desmin staining was performed, if not previously done, to strengthen the diagnosis of malignancy. Identical FFPE-cut sections from cell blocks used for diagnosis were stained with the PD-L1 antibody.

The study was approved by the Regional Ethical Board, Southern Health Care Region, Lund University, no. 2006/399.

**Immunocytochemistry**

**PD-L1 antibody**

PD-L1 IHC 28-8 pharmDx is a detection kit of PD-L1 with qualitative immuno-histochemical assay using monoclonal immunoglobulin G rabbit anti–PD-L1, clone 28-8 (Dako North America, Inc., Carpinteria, CA). The kit is intended for use to detect PD-L1 protein in FFPE tissue blocks using EnVision FLEX visualization system on Autostainer Link 48, AS48430 (Dako North America, Inc.) following the manufacturer’s protocol.

Target retrieval solution (Dako North America, Inc.) buffer was diluted 50 times with distilled water and preheated to 65°C using a Digital Decloaking Chamber, PT10030 (Dako Colorado, Inc., Fort Collins, CO). Heat-induced antigen retrieval was performed in the PT Link Pre-Treatment Module, PT10030 (Dako Colorado, Inc.) with target retrieval solution buffer for 20 minutes at 97°C at low pH. After 20 minutes, the buffer was cooled to 65°C, and the slides were taken out and subsequently washed for 5 minutes in wash buffer. Thereafter, the slides and reagents bottles were loaded in the Autostainer Link 48, AS48430 (Dako North America, Inc.). Before ICH automated processing, the washing buffer was added to each slide to prevent drying of the cut FFPE sections.

Immunostainings were run both during the day and overnight with automatic rehydration with distilled water to prevent drying, preventing the nonspecific binding of antibodies. Each block was cut twice; one was stained with antibodies, the other was stained with negative control...
reagent, the latter of which consists of immunoglobulin G antibodies that lack specificity against PD-L1 and works as an isotype control. Finally, the slides were covered with coverslips using permanent mounting media. All steps were performed according to manufacturer’s instruction.

**Evaluation of Immuno-cytochemical Staining**

The slides were assessed light microscopically, and PD-L1 expression in tumor cells was evaluated according to an assessment manual from Dako. Only tumor cells showing membrane staining were judged as PD-L1–positive. PD-L1 staining was indicated by a brown (3,3'-diaminobenzidine) reaction product, and positive PD-L1 staining was defined as complete surrounding and/or linear partial plasma membrane staining of tumor cells at any intensity. Cytoplasmic reactivity in malignant cells was not considered positive. Nonmalignant cells and immune cells such as lymphocytes or macrophages were not included in the assessment of PD-L1 reactivity.

The percentage of tumor cells exhibiting positive membrane staining at any intensity in the entire specimen was assessed as \(<1\%\) (negative), \(\geq1\%-5\%\), \(>5\%-10\%\), \(>10\%-50\%\), and \(>50\%\).

The scoring was performed blindly, initially by two authors (T.S. and M.S.I.M.) and by another author independently (K.D.), and cases with discrepant scores were then assessed by two authors (A.D. and M.S.I.M.) together to achieve consensus by manual counting of malignant cells and PD-L1–positive cells.

**Statistical Analysis of Data and Outcome**

The association of PD-L1 expression with patients’ characteristics was evaluated using the Student $t$ test for age and Fisher exact test for sex. Overall survival was determined and calculated from the date of the first specimen collection containing malignant cells until the date of death or the latest follow-up. Survival analysis for all patients and for actively treated patients grouped by PD-L1 expression at the 1% and 50% cutoff was performed using the Kaplan-Meier method and analyzed using a log-rank test. Three patients were right-censored. All $P$ values were determined using two-sided tests and $P > .05$ was not
RESULTS

A total of 74 patients with MM diagnosis were analyzed for PD-L1 expression. Thirteen cases had to be excluded, 11 due to an insufficient number of malignant cells or due to insufficient material left in the cell block. Two cases revealed positivity in scattered cells but contained <100 tumor cells, and were therefore excluded according to the recommendations by the manufacturer. Hence, a total of 61 cases, 47 histologically verified, with available cell blocks and adequate amount of cells were included in the analysis. In 5 of the included cases, four histologically verified, obviously malignant mesothelial cells had not been observed in the section and the diagnosis in the effusion had been strengthened by desmin staining. Of the analyzed cases, 23 of 61 were positive with a cutoff level of ≥1% and 6 of 61 with a cutoff level of >50%. At the 1% positivity level, the evaluation by T.S. and M.S.I.M. followed by that of K.D. agreed in 52 of the 61 cases. In 4 of the discrepant cases, the examiners disagreed as to whether the frequency of positive cells was <1% or ≥1%-5%. In 5 cases, one of the examiners considered the number of malignant cells insufficient (<100). At the cutoff level of 50%, there was disagreement only in 1 case, caused by weak staining. Images from samples illustrating different cell components and proportions of positive cells are shown in Figures (2 and 3), and 4, respectively.

Expression of PD-L1 as related to patients’ age, sex, active therapy, survival, and histological subtypes of MM, as well as the different levels of positivity, are shown in Table 1. There were no significant differences between patients with PD-L1 expression and without PD-L1 expression at either the 1% cutoff value or the 50% cutoff value according to age at diagnosis (P = .54, .23), sex (P = .24, .58), or survival (P = .16, .90). PD-L1 reactivity and survival pattern for the actively treated patients did not differ from the total material. Kaplan-Meier plots of survival at the 1% cutoff and 50% cutoff are shown in Figure 5.

DISCUSSION

There are a number of technical issues in which the quantification of PD-L1 expression in effusions differs from biopsies. The cellular content of effusions is complex and
the malignant cells are mixed with inflammatory cells and benign mesothelial cells that may give rise to false positivity. However, according to our preliminary tests, mesothelial cells in benign effusions did not exhibit PD-L1 positivity.

The analysis of malignant effusions may also be hampered by low overall cellularity and the malignant cells may be fewer compared with inflammatory background cells. As expected, cellular samples with predominance of malignant cells presented no interpretation problems (Fig. 2), but samples with an admixture of macrophages and fewer malignant cells, especially if they were dispersed, were slightly more difficult to assess. However, in all cases, these difficulties could be overcome using auxiliary immunocytochemical staining methods as described previously.

Another problem may be insufficient material available in the cell block. In our study, 13 of 74 (18%) effusions had to be excluded, in 2 cases due to a malignant cell number <100 and in 11 cases due to insufficient cell block material.

We found PD-L1 expression in 23 of 61 (38%) MM cases. In previous studies, based on histological specimens, the reported proportion of positive cases has varied between 17% and 63%.25–27,32,33 Thus, our results fall well within the range of expected positivity.

There are several explanations for the considerable variation regarding the positivity rate between different studies. One reason is that different criteria for the definition of positivity have been used in the different studies. Some studies also include intensity as a criterion. Furthermore, the threshold for positivity has been varying. The threshold often used is 1%,26,27,32 but some studies used a threshold of 5%.25,33 We based our evaluation on the criteria recommended by the manufacturer: only membranous, partial, or circumferential staining in tumor cells was considered as positivity, and reactivity was graded as the proportion of tumor cells stained.31

The manufacturer’s evaluation of clinical utility of the antibody that we tested was based on a randomized study of patients with non–small cell lung cancer in which there was no difference in response to treatment with the antibody between ≥1%, ≥5%, and ≥10% levels of reactivity.34 Thus, a cutoff value for clinical use of ≥1% positive cell is recommended. No such study with this
The antibody has been performed previously on MM, and consequently, there is no relatable study of therapy response.

The frequency of PD-L1–positive cells in the different histological types of malignant effusion cells in our material is shown in Table 1. An association between PD-L1 reactivity and histological tumor subtype of MM has been reported previously, the expression being higher in sarcomatoid MM. The proportion of sarcomatoid MM in our material, 7%, is lower than the proportion generally reported in the literature or in studies that have investigated PD-L1 in histological material. This low proportion is explained by the fact that our basic inclusion criterion was an effusion containing diagnostic malignant cells. Although sarcomatoid MM gives rise to effusions, it seldom sheds malignant cells into the effusion. In addition, malignant cells in effusions may acquire epithelial characteristics.

In our study, a tendency (though not statistically significant) toward longer survival was associated with positivity for PD-L1 at the 1% threshold for positivity. This is in contrast to previously reported histology-based studies of MM in which PD-L1–positive samples were associated with shorter survival. It is, however, noteworthy that in other types of tumors (eg, non–small cell lung cancer and colorectal cancer), the reverse is true. In our study, PD-L1–positive cases were proportionally represented (8/23) among patients who had been selected for active therapy after the initial diagnosis, and the statistically nonsignificant tendency toward better survival of patients with PD-L1–positive tumor cells was similar.

The relationship between PD-L1 expression and cancer biology is complex. Tumor stage, histological subtype, and biological variability can explain the differences and the strong variability in patient survival and therapy response. Our material was obtained early in the disease history, concurrently with diagnosis, and thus before any treatment. In the reported biopsy-based data, some proportion of the patients had been in treatment before the biopsy. According to one study, chemotherapy seems to eliminate PD-L1 from cancer cells and could thus make the cases appear PD-L1–negative and skew the apparent outcome.

As shown for many cancer types, the immunological characteristics of different types of tumor tissue (eg, primary tumors, metastatic tumors, circulating tumor cells,
and effusions) may differ, and there might even be differences within the same tumor manifestation.\textsuperscript{15} For MM, the only published data thus far included 7 cases with matching biopsies and effusions.\textsuperscript{33} However, the outcome reported in that study is not directly comparable to our results due to differences in the type of effusion, technique, and assessment criteria applied. However, the key issue is not the correspondence between effusion-based and biopsy characterization of PD-L1 positivity, or even the positivity as such. For MM, it could even be the fraction of malignant cells in effusions that show positivity, which can vary widely as shown in Table 1; some of our samples showed over 50% positive malignant cells. The relevant question is what criteria can predict the success of anti–PD-L1–based therapies for MM. Because MM is relatively rare, the answers can only be obtained in a large multicenter study in which the parameters might include not only the side-by-side comparison of the utility of biopsies and effusions, but also the different detection antibodies and the scoring criteria including both the positive cell numbers and the cell types to consider.

### Table 1. Expression of PD-L1 Correlated to Patients’ Characteristics, Survival, Treatment, and Histological Subtypes of MM

| Characteristics                  | Negatives | Positives | P Positives/Negatives |
|----------------------------------|-----------|-----------|-----------------------|
|                                  | All Patients | <1% | ≤50% | >50% | ≥1% | 50% Cutoff | 1% Cutoff |
| Median age at diagnosis, y       | 73 | 72.7 | 72.8 | 76.7 | 73.2 | .23 | .54 |
| Median survival, mo              | 13 | 10 | 13 | 16 | 16 | .90 | .16 |
| Sex, n (%)                       | Male | 53 (87) | 31 (82) | 47 (85) | 6 (100) | 22 (96) | .58 | .24 |
|                                  | Female | 8 (13) | 7 (18) | 8 (15) | 0 (0) | 1 (4) |               |
| Active treatment, n (%)          | Male | 23 (38) | 15 (39) | 21 (38) | 2 (33) | 8 (35) | .83 | .71 |
| Median age at diagnosis, y       | 70.7 | 70.8 | 70.7 | 69.5 | 68.2 |               |
| Median survival, mo              | 14 | 13 | 14 | 17 | 18 | .48 | .39 |
| Histology, n (%)                 | Epithelioid | 37 (61) | 25 (66) | 4 (44) | 3 (75) | 2 (50) | 3 (50) | 12 (52) |
|                                  | Mixed | 4 (7) | 2 (22) | 1 (25) | 1 (25) | — | 4 (17) |
|                                  | Sarcomatoid | 3 (5) | 2 (5) | — | — | 1 (17) | 1 (4) |
|                                  | Unknown | 17 (28) | 11 (29) | 3 (33) | 1 (25) | 2 (33) | 6 (26) |
| Total, n (%)                     | 61 (100) | 38 (100) | 9 (100) | 4 (100) | 4 (100) | 6 (100) | 23 (100) |

**Figure 5.** Kaplan-Meier survival plots of 61 MM patients grouped by PD-L1 expression at the 1% cutoff (left, \( P = .16 \) [log-rank]) and the 50% cutoff (right, \( P = .90 \) [log-rank]).
In conclusion, we have demonstrated that an evaluation of the expression of PD-L1 made on cell blocks from mesothelioma effusions is feasible and the results are comparable to those reported previously in studies based on biopsies. Effusions accompany practically all cases of MM and effusion cytology enables a diagnosis in most cases of epithelial and mixed MM and can be the earliest diagnostic tool for the disease. If confirmed, effusion-based characterization of susceptibility to anti–PD-L1 therapy could facilitate earlier therapy and thus therapeutic response with less patient inconvenience, and at a lower cost.

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CONFLICTS OF INTEREST DISCLOSURES

The authors made no disclosure.

AUTHOR CONTRIBUTIONS

Mohammed S. I. Mansour: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing (original draft), writing (review and editing), visualization, project administration, funding acquisition. Tomas Seidal: conceptualization, methodology, validation, investigation, resources, writing (original draft), writing (review and editing), supervision, funding acquisition. Ulrich Mager: conceptualization, methodology, validation, investigation, resources, writing (review and editing), visualization, funding acquisition. Amir Baigi: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing (original draft), writing (review and editing). Katalin Dobra: conceptualization, validation, investigation, writing (review and editing), supervision, project administration, funding acquisition.

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