Effusion cytology of malignant mesothelioma enables earlier diagnosis and recognizes patients with better prognosis

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Abstract
A conclusive diagnosis of malignant mesothelioma (MM) can be based on effusion cytology using the guidelines for the cytopathologic diagnosis of epithelioid and mixed-type MM. Briefly, the diagnosis is obtained when the mesothelial phenotype of malignant cells is established by ancillary techniques. This study is based on the comparison of the overall survival rates of patients with MM when diagnosed by effusion cytology, histopathology, or a combination of both. A total of 144 patients were diagnosed with epithelioid and mixed-type pleural MM at Karolinska University Hospital between 2004 and 2013. The diagnosis was obtained by histopathology in 74 cases and by cytological examination of pleural effusion in 70 cases. In 29 of the latter cases, a diagnostic biopsy was obtained simultaneously. A total of 104 patients received chemotherapy. All diagnoses were supported by clinical findings, including computer tomography scans. The median time between first symptoms and diagnosis was similar for cytology and histopathology. However, a delay of more than 6 months after first symptoms was seen in many patients in the histopathology group, resulting in late onset of treatment. The overall survival and proportion of long-term survival were significantly better for cases diagnosed by cytology. Similarly, a better survival, following a cytological diagnosis, was also seen in patients who were only provided the best supportive care. Accurate cytological diagnosis enables conclusive diagnosis of MM. Our finding enables the initiation of treatment as soon as the cytological diagnosis is established, avoiding further delay and deterioration of patient survival and possibilities for treatment.

KEYWORDS
cytology, effusion, histology, malignant mesothelioma, survival time

1 | INTRODUCTION

Malignant mesothelioma (MM) is a highly aggressive tumor of the serosal cavities, with a median survival of 4 to 12 months.1,2 The most common location of MM is pleural, malignant pleural mesothelioma (MPM). The incidence of MPM has increased markedly over the past two decades and is expected to peak in 2020 to 2025.3-5 MM occurs after a long latency period after the first asbestos exposure. This delay has been attributed to the slow progression of the disease, and the development of symptoms is often at a late stage. The symptoms include pain, dyspnea, and pleural effusion. The diagnosis of MM is often challenging, and the use of effusion cytology can provide a definitive diagnosis.
Treatment options are, however, limited because the diagnosis is typically established late during the course of the disease. The first symptom of the disease is often accumulation of fluid in the pleura, so called effusion, which is withdrawn for therapeutic reasons allowing the expansion of the lungs, at the same time becoming the earliest available biological material for diagnosis. Studies have shown that early treatment improves patient survival, thus a diagnosis should be reached as quickly as possible, avoiding unnecessary delays.

Traditionally, the diagnosis of MM often depends on histopathology, as has been the gold standard for decades. Recent audits, however, showed that effusion cytology provides an accurate option for this diagnosis, with a positive predictive value of 100% and a sensitivity of approximately 70%. International guidelines for the cytopathologic diagnosis of epithelioid and mixed-type MM have been published, initiated by the International Mesothelioma Interest Group, and endorsed by the International Academy of Cytology and Papanicolaou Society of Cytopathology.

Briefly, the cytological diagnosis comprises two steps: first to establish that the cells are malignant and then to show that the malignant cells are mesothelial in origin. Malignancy can be based on conventional cytological criteria such as numerous cell groups and nuclear polymorphism. Sometimes, however, MM cells show only mild atypia, indistinguishable from reactive mesothelium with routine stains. In these cases, the sensitivity is greatly enhanced by ancillary analyses such as immunocytochemistry and ploidy analysis by fluorescence in situ hybridization (FISH). In fact, whenever an effusion is rich in mesothelial cells, the possibility of an MM should be kept in mind.

Once malignancy is established, ancillary analyses will define the tumor phenotype. Diagnostic criteria for immunohisto- and cytchemistry are in fact the same here, considering that some of the stains work better on formalin-fixed and paraffin-imbedded tissue than on the alcohol-fixed exfoliative material and vice versa. Cells of mesothelial origin can be demonstrated by calretinin, mesothelin, HBME-1, podoplanin (D2-40), and WT-1, while the diagnosis can be excluded by CEA, BerEp-4, MOC-31, TTF-1, and Pax8. BerEp-4 and MOC-31 label the same epithelial cell adhesion molecule complex, the former performing somewhat better in nonfixed material and the latter following formalin fixation. Both histology and cytology guidelines state that two markers in favor of mesothelioma and two excluding the diagnosis are sufficient. With disputable staining results, however, additional antibodies must be used. These reactions can also be complemented by analyzing soluble markers such as hyaluronan and mesothelin. In case there is still a suspicion for mesothelioma, electron microscopy can be helpful, providing that the sample contains cell groups and that cells have been fixed properly early in the process.

The aim of the present investigation was to evaluate the clinical outcome of patients with epithelioid and mixed-type MPM, comparing patients with the first conclusive diagnosis obtained by effusion cytology and those with the first conclusive diagnosis obtained by histopathology, including the possible influence of MM phenotype, gender, age, known asbestos exposure, signs of inflammatory response, biomarker levels, and time from first sign of disease to diagnosis and treatment.

2 MATERIALS AND METHODS

2.1 Study design and patient selection

This is a retrospective observational study evaluating the diagnostic and prognostic values of the cytological and histological diagnosis of MM and summarizes 10 years of clinical experience in praxis. A total of 189 patients were diagnosed with pleural MM in Stockholm County between January 2004 and December 2013. Clinicopathological characteristics were retrieved from the cytology/pathology database at the Department of Clinical Pathology and Cytology, Karolinska University Laboratories, Stockholm. Only patients with pleural mesothelioma who did not have other malignant lethal disease at the time of the diagnosis were included (181 cases). Tumors with sarcomatoid phenotype (26 cases) were excluded because they will not be detected by cytology due to limited cell shedding. Another 11 patients were excluded because their diagnoses were obtained by fine-needle biopsies, leaving a total of 144 cases with epithelioid or mixed-type MM diagnosed either by biopsy or by effusion cytology (Figure 1).
2.2 | Clinical characteristics

All patients had radiological and clinical evidence of MM or developed such evidence during the clinical course. Clinical information on treatments, therapeutic responses, and survival time was obtained from the Stockholm County clinical database. Patients were categorized into two groups based on whether they received chemotherapy. The material was further classified based on the initial diagnosis, resulting in three categories: cytology alone, histopathology alone, or a combination of both within a time interval of 1 month.

Of the 104 patients who received therapy, the diagnosis was based on histology in 57 cases, by cytology only in 25 cases, and there were simultaneous cytological and histological diagnoses available in 22 cases (Table 1). In many of these cases, the thoracentesis and biopsy sampling were performed in one session, and for a few others, histology was a follow-up due to reluctance as to the reliability of the cytological diagnosis. Most patients received combination therapy consisting of carboplatin and pemetrexed, with no difference between cytologically and histologically diagnosed tumors. Depending on the clinical condition, a few patients in each group only received one of the drugs. Forty patients (18 in the histology group, 15 in the cytology group, and 7 diagnosed by both techniques simultaneously) received the best supportive care.

2.3 | Pathology

Morphological reassessment verified that all diagnoses met postulated diagnostic criteria. All histological diagnoses were based on biopsies obtained during thoracoscopy or by transthoracic needle sampling. The morphology was supported in all cases by the routine immunohistochemistry available during this period as described in published guidelines, principally using the same antibodies as recommended for the histopathological diagnosis. Immunocytochemistry on effusions were in all cases performed cytospin preparations without formalin fixation. Thus, among cytological findings supporting malignancy, there is richness in papillary groups with reactivity to epithelial membrane antigen (EMA) but not desmin (Figure 2). As described in the guidelines, reactions with desmin perform somewhat better that the more recently recommended BAP-1 when performed on nonfixed cells, while the latter is to prefer formalin-fixed cell blocks. The mesothelial phenotype is shown by the presence of calretinin and mesothelin with simultaneous absence of BerEp4 and CEA (Figure 3). The cytological diagnoses were based on cells recovered from effusions. The cytomorphology was supported by immunocytochemical demonstration of at least four immunomarkers as recommended by the literature. Immunocytochemical demonstration of additional biomarkers, hyaluronan and mesothelin, was performed in 28 cases. The diagnosis was further supported by electron microscopy of the cell pellet in 34 cases. Ploidy analysis by FISH (UroVysion, Abbot) was used to support the malignant nature of the cytological specimen in 14 cases. The cytological diagnoses were in accordance with radiological findings and clinical course in all cases. The performance of the employed diagnostic routines has been described, showing similar sensitivity and positive predictive values as shown in other experienced laboratories.

An abundance of inflammatory cells in an effusion may not only hinder a cytological diagnosis by masking the diagnostic tumor cells but also influence tumor proliferation, potentially influencing the clinical outcome. To evaluate this, the proportion of such cells in 32 nondiagnostic effusions available from the histology-only group were re-evaluated by two cytopathologists (SAO and AH) studying the possible influence on prognosis.

2.4 | Statistical analysis

Overall survival was demonstrated by Kaplan-Meier survival curves, measuring times from the date of diagnosis. The shortest follow-up time of a still alive patient was 48 months. Group differences were tested using a log-rank test. The importance of possible confounding factors among the treated patients, including age, sex, sensitivity to

| TABLE 1 | Patient characteristics |
|----------|-------------------------|
|          | Cytology | Histology | P value  |
| N        | 47       | 57        |          |
| Age, median (IQR) | 69 (64, 76) | 69 (64, 73) | .51 |
| Time from symptom to diagnosis (months), median (IQR) | 0 (0, 1) | 1 (0, 6) | .001* |
| Time from diagnosis to treatment (months), median (IQR) | 2 (1, 3) | 1 (1, 2) | .003* |
| Time from symptom to treatment (months), median (IQR) | 3 (2, 4) | 3 (2, 6) | .21 |
| Sex       |          |          |          |
| Male      | 41 (87%) | 46 (81%) | .37     |
| Female    | 6 (13%)  | 11 (19%) |          |
| Asbestos exposure |          |          |          |
| Known     | 30 (64%) | 30 (53%) | .25     |
| Unknown   | 17 (36%) | 27 (47%) |          |
| Treatment response |          |          | .73     |
| Progressive disease | 14 (30%) | 20 (35%) |          |
| Stable disease   | 27 (57%) | 32 (56%) |          |
| Partial response | 6 (13%)  | 5 (9%)   |          |

Note: Asterisk denotes statistically significant difference.
therapy, and known asbestos exposure, was evaluated by univariable and multivariable Cox regression. A separate multivariable analysis evaluated the importance of time from diagnosis and chemotherapy when modeling the survival time from chemotherapy.

To visualize the possible influence of the histological type, cases with simultaneous cytopathologic and histopathological diagnoses were compared regarding the proportion of sarcomatoid tumor component in the histological material, using cut-off levels of both 10% and 20%. To evaluate the contribution of morphological features to patient survival, we studied the presence of papillary structures in pleural biopsies as a cause for recognizable diagnostic findings in an effusion. The importance of such a phenotype was studied by comparing survival over time in cases detected only by histology and cases with simultaneous cytology and histology. The importance of such factors, which were only available in a subset of patients, was analyzed by a rank-sum test for continuous variables and a χ² test for categorical variables. The statistical analyses were performed using Stata (v.13).

This study was approved by the local ethical committee (4/122007/1089-32 and 2009/1138-31/3).

3 | RESULTS

Based on the average time lag between the first sign of disease and diagnosis, a histological diagnosis was obtained 30 days later than a cytological diagnosis (Table 1; P = .001). This time varied considerably in the histology group, as the diagnosis was obtained after more than 6 months in 25% of the cases. The time from diagnosis to treatment was 1 month shorter following a histological diagnosis than a cytological diagnosis (P = .003), resulting in similar times between first sign and treatment for both groups (P = .21). In the present material, the cytological diagnosis of MM was obtained with a complete sensitivity of 86% and the absolute sensitivity (definitive diagnoses only) was 60%. As all patients presented or developed radiological and clinical
MM, the positive predictive value of cytology was 100%, that is, there was no false-positive diagnosis during the 10 years studied. However, the earlier cytological diagnosis did not correlate with earlier treatment in this material. During the first 6 months, the overall survival after diagnosis for patients first diagnosed by effusion cytology was slightly prolonged compared with those diagnosed by histopathology, corresponding to the natural course with an earlier diagnosis (Figure 4A). However, the Kaplan-Maier plots deviated significantly after 10 to 12 months. The overall survival was significantly better in patients in whom the diagnosis was obtained with effusion cytology compared with those in whom the diagnosis was based on histopathology alone, with median survival values of 20 months and 14 months, respectively (P = .02). The 3-year survival was 31% and 11% for the two groups, respectively, and the corresponding 4-year survival was 19% and 5%, respectively.

Better overall survival in the cytology group was also found in patients not receiving chemotherapy (P = .002; Figure 4B), with a median survival of 9 months following a cytological diagnosis and 3 months following a histopathological diagnosis. When given, treatment increased survival by 11 months in the cytology group but only 4 months in the histology group. The survival curves for patients with simultaneous histological and cytological diagnoses and those with first diagnosis based on cytology only follow each other closely and differ significantly from those in whom the diagnosis was based on histopathology alone (P = .05, Figure 5). When confined to the subgroup with histologically verified epithelioid morphology (Figure 6), the difference in median survival was still 5 months, although statistically not significant with the fewer cases (19 and 14 months for the two groups, respectively; P = .08).

**FIGURE 3** The mesothelial lineage of the tumor cells is demonstrated immunologically. Calretinin (G,H) and mesothelin (I,J) as well as absence of BerEp4 (K,L) and CEA (M,N) fulfill the minimal requirements of two in favor of and excluding such differentiation (×100) [Color figure can be viewed at wileyonlinelibrary.com]
As presented earlier, chemotherapy was given not only earlier, but also slightly more often, to patients diagnosed histologically (cytology only 26/41, 63%; simultaneous cytology and histology 22/29, 76%; histology only 56/74, 76%), but this difference was not significant when comparing cytology with histology only ($P = .20$). Survival correlated with the responses to therapy in both cytologically and histologically diagnosed cases (Table 2). Although slightly better in cases detected by cytology, this does not contribute to the improved survival of cytologically diagnosed cases, as earlier diagnosis by cytology was not followed by earlier treatment.

Among the 104 patients given chemotherapy, 87 were men and 17 women, with no correlation with the means of diagnosis ($P = .37$; Table 1). Asbestos exposure could be confirmed in only 50 cases, slightly more common among cases with a first cytological diagnosis (65% vs 52%; $P = .25$). Biomarker analyses (hyaluronan and/or mesothelin) had been performed in 35 patients, 32 of whom had elevated levels. The histological tumor phenotype could be confirmed in 82 cases, with mixed type found in 7/24 (29%) cytologically recognized cases, the same proportion as in tumors with only a histological diagnosis (17/58, 29%).

Among 32 cases with nondiagnostic effusions (MM diagnosis obtained by histology), 19 presented an abundant admixture of inflammatory cells, leaving 13 cases with fewer such cells. The presence of inflammatory cells did not correlate with survival; the mean survival was 12 months for both groups. The presence of papillary elements is obviously more common in cases diagnosed by cytology compared with cases only diagnosed by histology (79% vs 31%, $P = .005$). However, papillary differentiation had no significant influence on overall survival.
survival and could not explain improved survival rates associated with cytologically obtained diagnoses. Biomarker levels (hyaluronan and mesothelin) were measured mainly in the cytology group; only 2 cases in the histology group had an effusion in which these parameters were analyzed. The registered parameters did not differ significantly between the two methods of diagnosing mesothelioma.

Cox regression (Table 2) showed that the method of diagnosis \((P = .034)\) and sex \((P = .040)\) were significant factors in survival after diagnosis. Thus, patients with cytologically recognizable tumors had better overall survival. Although the cytological diagnosis was obtained 1 month earlier, the difference in median survival after onset of therapy was still 6 months (19 and 13 months for cytology and histology, respectively, \(P = .055\)).

### Table 2 Cox regression influencing the survival of malignant mesothelioma patient

| Variable               | Crude Hazard ratio (95% CI) | P value | Adjusted Hazard ratio (95% CI) | P value |
|------------------------|----------------------------|---------|-------------------------------|---------|
| Type of diagnosis      |                            |         |                               |         |
| Cytology               | 1 (ref.)                   |         |                               |         |
| Histology              | 1.61 (1.06-2.45)           | .026    | 1.59 (1.04-2.44)              | .034    |
| Sex                    |                            |         |                               |         |
| Male                   | 1 (ref.)                   |         |                               |         |
| Female                 | 0.61 (0.34-1.09)           | .095    | 0.52 (0.28-0.97)              | .040    |
| Age                    |                            |         |                               |         |
| Known                  | 1.00 (0.97-1.03)           | .854    | 1.00 (0.97-1.03)              | .951    |
| Unknown                |                            |         |                               |         |
| Asbestos exposure      |                            |         |                               |         |
| Known                  | 1 (ref.)                   |         |                               |         |
| Unknown                | 1.03 (0.68-1.57)           | .873    | 1.08 (0.67-1.76)              | .744    |
| Treatment response     |                            |         |                               |         |
| Progressive disease    | 1 (ref.)                   |         |                               |         |
| Stable disease         | 0.48 (0.31-0.75)           | .001    | 0.48 (0.30-0.77)              | .002    |
| Partial response       | 0.38 (0.18-0.80)           | .011    | 0.43 (0.20-0.94)              | .034    |

### 4 Discussion

The diagnosis of MM has traditionally required histological material. However, this implies invasive approaches, such as core needle biopsy or thoracoscopy. The cytological examination of an effusion has also proven to be diagnostic in a substantial proportion of cases. It is sometimes claimed that cytology is unable to demonstrate invasiveness and therefore insufficient for a conclusive diagnosis of MM. Cytology, however, can recognize malignant conditions based on other criteria such as homozygous deletion of the 9p21 band, biomarker analyses, and electron microscopy. This reliability has previously been repeatedly shown.

The presence of an effusion is often the first manifestation of this tumor. This fluid is drained by thoracentesis to relieve symptoms, resulting in the first material available for diagnosis. The diagnosis requires immunocytochemistry with the same markers as used in histology. When effusion cytology in this way can demonstrate the presence of MM, the diagnosis will be obtained at a somewhat earlier stage. A biopsy is often obtained later, often with a delay of 1 month (interquartile range 0-6 months), which may decrease the effect of chemotherapy.

The survival time was significantly better in patients in whom the diagnosis was obtained by cytology compared with histologically diagnosed patients. The survival curves run closely for the first few months, but their later courses diverge and differ significantly. Although patients with histologically recognized tumors survived as expected for this tumor group, the median survival was improved by 6 months in cytologically diagnosed cases, with 19% of these latter patients still alive after 4 years compared with only 5% in the histology group. During the study, survival data were published from a large Australian material. In that study, cases diagnosed cytologically, which includes both epithelioid and mixed-type MMs, had similar overall survival rates as histologically diagnosed purely epithelioid MMs and better than histologically mixed MMs, which is in accordance with our results.

This improved survival, which is substantial in our study, was not the result of earlier treatment, as the gain in time for diagnosis was lost due to later onset of therapy following a cytological diagnosis. In fact, the gain in time for diagnosis was of the same magnitude as a delay in initiating treatment. Furthermore, although untreated patients with cytologically diagnosed tumors present shorter survival than those given chemotherapy, they still had better overall survival than the nontreated group with a first diagnosis based on biopsy only. The subgroup of patients in which a diagnostic biopsy was obtained simultaneously with a diagnostic effusion had an overall survival almost identical to patients with the first diagnosis based on cytology only and differing significantly from the patients with only a histological diagnosis. Thus, the less favorable outcome after a histological diagnosis does not depend on complications attributed to the surgical sampling procedure.

In the present study, the cases that were recognized by cytology did not obtain treatment earlier; thus, the better prognosis depends on other factors than earlier onset of therapy. Many of the differences between cytologically and histologically diagnosed cases seem to persist when confined to purely epithelioid MMs although not statistically significant \((P = .078)\). The proportion of mixed-type MMs, with more than 10% or 20% sarcomatoid differentiation, could not explain...
the different outcomes between cytologically and histologically diagnosed tumors, that is, there was no indication that the difference in survival depended on the differences in proportions of sarcomatoid differentiation.

Cytologically recognized MMMs often show papillary differentiation. This phenotype is, however, considered more indolent,26-28 and could not explain the improved survival. In a recent study, integrated molecular pathology could demonstrate that six clusters of genetic aberration in MM showed distinct survival outcomes.29 Whether any of these subgroups correlate to exfoliation of recognizable mesothelioma cells and better outcome in the present material remains to be shown. It should be noted that none of the included cases were diagnosed with specific entity "Well Differentiated Pleural Mesothelioma" (WDPM). The better outcome could not thus be correlated to morphological or molecular phenotype. A slightly better survival of female patients has also been shown previously30,31 and may relate to certain differentiated MM types that are less associated with asbestos exposure and, thus, relatively more common in women.

Taken together, the results show that effusion cytology identifies a large subgroup of mesothelioma patients with better prognosis that often respond to chemotherapy. Explanations for this better outcome may correlate to the differentiation of the tumor, as well as to its ability to exfoliate and be recognized, and the molecular background is still not known. As this effusion is often an early sign of the disease, it enables an earlier diagnosis. The diagnostic procedure presented here followed the international guidelines, and the positive predictive value obtained for the cytological diagnosis is sufficient to motivate treatment without further delay. In case the earlier diagnosis obtained by effusion cytology also results in earlier onset of treatment; further, improved survival would be expected.8

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