Using Immunohistochemistry to Assess the Accuracy of Histomorphologic Diagnosis of Aspergillosis and Mucormycosis

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Background. Data on the accuracy of conventional histomorphologic diagnosis are limited, especially in mucormycosis. We therefore investigated the accuracy of histomorphologic diagnosis of mucormycosis and aspergillosis, using immunohistochemistry (IHC) tests for mucormycosis and aspergillosis.

Methods. Patients enrolled met the modified criteria for proven and probable mucormycosis (during a 22-year period) or invasive aspergillosis (during a 6-year period) and had formalin-fixed, paraffin-embedded tissues available. We first tested the diagnostic performance of IHC for mucormycosis and aspergillosis in proven cases. Then we determined the accuracy of histomorphologic diagnosis of probable cases, using the IHC tests.

Results. In 7 proven cases of mucormycosis, the sensitivity and specificity of mucormycosis IHC were 100% (95% confidence interval, 65%–100%) and 100% (68%–100%), respectively. In 8 proven cases of aspergillosis, and the sensitivity and specificity of aspergillosis IHC staining were 87% (53%–98%) and 100% (65%–100%), respectively. Of 23 probable mucormycosis cases, 20 (87%) were positive with mucormycosis IHC, 2 (9%) were positive with aspergillosis IHC (including 1 positive for both), and 2 were negative with both. Of 16 probable aspergillosis cases, 10 (63%) were positive with aspergillosis IHC, 4 (25%) were positive with mucormycosis IHC, and 2 (13%) were negative with both tests.

Conclusions. Aspergillosis and mucormycosis seem not to be correctly diagnosed morphologically, because some of the probable cases showed either test with both antibodies or failure to stain with the homologous antibody. In the absence of fungal culture results, the IHC tests seem helpful in differentiating between aspergillosis and mucormycosis.

Keywords. mucormycosis; aspergillosis; histomorphology; immunohistochemistry.

The incidence of systemic fungal infections parallels the increased use of immunosuppressive therapy, steroids, and cytotoxic drugs [1]. The prevalence of mucormycosis, in particular, has been increasing along with the increasing use of antifungal agents with anti-Aspergillus activity, such as voriconazole [2, 3] and it is the third most common fungal infection after candidiasis and aspergillosis [4]. In addition, because voriconazole has no activity in mucormycosis but results in better survival of patients with invasive pulmonary aspergillosis (IPA) than amphotericin B [5, 6], distinguishing between IPA and pulmonary mucormycosis (PM) is important clinically. Although definite diagnoses can be made by combining histomorphologic diagnosis with fungal culture results, cultures are often negative, especially for mucormycosis [1]. In this situation, clinical diagnosis and treatment rely heavily on histomorphologic diagnosis. However, definitive diagnosis is not always possible based on the morphology of the fungal elements within tissues because of morphologic similarities between the tissue forms of
several fungal genera, and the frequent presence of sparse or atypical fungal elements [7]. We therefore evaluated the accuracy of mucormycosis and aspergillosis immunohistochemistry (IHC) tests and then used these tests to determine the accuracy of histomorphologic diagnoses of aspergillosis and mucormycosis.

METHODS

Study Population and Design
This study was performed at the Asan Medical Center, a 2700-bed tertiary care teaching hospital in Seoul, South Korea. The medical records of adult patients (≥16 years old) who met the criteria for proven mucormycosis and probable PM from January 1992 to December 2013 were retrospectively reviewed. The incidence of invasive aspergillosis is known to be 4–5-fold higher than that of mucormycosis [8, 9]. We therefore estimated that the number of cases of invasive aspergillosis during the last 6 years would be similar to the number of mucormycosis cases during the 22-year study period. Therefore, all patients who met the criteria of proven aspergillosis and probable IPA from January 2008 to December 2013 were also retrospectively reviewed. Because the galactomannan (GM) assay (Platelia Aspergillus enzyme immunoassay [Bio-Rad]; GM index considered positive at ≥0.5) was available in our hospital from 2008, GM results were available for the invasive aspergillosis and mucormycosis cases enrolled between 2008 and 2013. Among the patients who met the above criteria, the ones enrolled in the study included those with formalin-fixed, paraffin-embedded specimens. We obtained the original diagnoses from the pathology reports.

In the first part of the study we tested the diagnostic performance of mucormycosis and aspergillosis IHC tests in proven mucormycosis and aspergillosis. To test the cross-reactivity of these 2 antibodies against other fungal infections (ie, candidiasis, fusariosis, and scedosporiosis) and nonfungal infection (ie, tuberculosis), we performed IHC on tissue specimens from patients with culture-proven candidiasis, fusariosis, and scedosporiosis between 1992 and 2013 and culture-proven tuberculosis between 2008 and 2013. We also checked the reactivity of the IHC stain on formalin-fixed, paraffin embedded pure cultures of Candida albicans, Aspergillus fumigatus, Aspergillus nidulans, Rhizopus stolonifer, Cunninghamella bertholletiae, Penicillium chrysogenum, Fusarium solani, and Scedosporium apiospermum.

In the second part of the study we determined the accuracy of histomorphologic diagnosis of probable PM and IPA using the IHC tests. We also examined the level of agreement of histomorphologic diagnosis between 2 experienced pathologists. Two independent and experienced pathologists (Y. S. P. and J. S. S., with 17 and 13 years’ experience in lung pathology, respectively) reviewed all patients with aspergillosis and mucormycosis without knowing the results of aspergillosis and mucormycosis IHC and fungal culture. The study protocol was approved by our hospital’s institutional review board.

Definitions
We used the modified criteria of the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) [10] because tissue invasion by fungal hyphae is considered definitive evidence for invasive fungal disease, irrespective of host factors. We therefore further classified invasive fungal disease as proven by the mycologic evidence in sterile culture—positive cases and as probable in nonsterile culture—positive cases or if there was no mycologic evidence except the GM assay. Briefly, proven mucormycosis was defined as histologic evidence of tissue invasion including nonseptated, right angle–branching filamentous fungi plus recovery of agents of mucormycosis (Rhizopus spp., Cunninghamella spp., Apophysomyces spp., Saksenaea spp., Absidia spp., Mucor spp.) by culture from sterile specimens. Probable PM was defined as the above histologic evidence with or without recovery of agents of mucormycosis by culture from nonsterile specimens, such as sputum or bronchoalveolar lavage (BAL) fluid. Proven IPA was defined as histologic evidence of tissue invasion including septated, acutely branching filamentous fungi plus recovery of Aspergillus species by culture from sterile specimens. Probable IPA was defined as the above histologic evidence plus recovery of Aspergillus species by culture from nonsterile specimens, such as sputum and/or BAL fluid, or positive GM assays.

IHC
IHC was performed on formalin-fixed, paraffin-embedded tissue sections using either a manual (for mucormycosis) or an automatic IHC device (for aspergillosis, Benchmark XT; Ventana Medical Systems). We used the manual protocol for mucormycosis IHC because it yielded better staining quality than the automatic protocol. Briefly, 4-µm-thick whole tissue sections were transferred onto poly-L-lysine–coated adhesive slides and dried at 74°C for 30 minutes. Epitopes were retrieved by applying steam at 96°C either for 60 minutes in 10 mmol/L citrate buffer (pH 6.0) for manual staining or for 1 hour in ethylene diamine tetraacetic acid (pH 8.0) in an autostainer. Samples were incubated with anti–Rhizopus arrhizus mouse monoclonal antibody (1:100; clone WSSA-RA-1; LSBio) for 1 hour at room temperature or anti-Aspergillus mouse monoclonal antibody (1:50; clone WF-AF-1; LSBio) in the autostainer. Sections were subsequently incubated with the Dako Envision System Kit (K5007; Dako) for 30 minutes and then developed with 3,3’-diaminobenzidine (DAB) solution (Dako) for manual staining or with an optiView Universal DAB kit (Ventana Medical Systems) in the autostainer. Slides were counterstained with Harris hematoxylin.
Statistical Analysis
Diagnostic performance was expressed in terms of sensitivity and specificity. Concordance between the 2 pathologists was assessed using the statistic. All tests of significance were 2 tailed, and differences were considered statistically significant at $P < .05$. Calculations were performed using the SPSS for Windows software package (version 21 K; SPSS).

RESULTS
Study Characteristics
During the study period, we identified 30 patients with mucormycosis for whom formalin-fixed, paraffin-embedded tissues were available; they included 7 patients with proven mucormycosis and 23 with probable PM. We also identified 24 patients with aspergillosis for whom formalin-fixed, paraffin-embedded tissues were available, including 8 patients with proven aspergillosis and 16 with probable IPA. Detailed clinical data and diagnoses are shown in Supplementary Tables 1–3.

Diagnostic Performance of IHC
We tested the diagnostic performance of mucormycosis and aspergillosis IHC in the 7 patients with proven mucormycosis and 8 with proven aspergillosis. Typical IHC findings are shown in Figure 1. The sensitivity and specificity were 100% (95% confidence interval, 65%–100%) and 100% (68%–100%), respectively, for mucormycosis and 87% (53%–98%) and 100% (65%–100%) for aspergillosis IHC (Table 1).

We tested the cross-reactivity of the mucormycosis and aspergillosis IHC in 2 cases of proven candidiasis, 7 of proven fusariosis, 2 of proven scedosporiosis, and 4 of tuberculosis. None of these cases were positive for mucormycosis or aspergillosis IHC. We also checked the reactivity of IHC in formalin-fixed, paraffin-embedded pure cultures of various fungal organisms (Supplementary Figure 1). Results of aspergillosis and mucormycosis IHC, respectively, were as follows for some of the pure cultures: A. fumigatus, positive and weak positive; A. nidulans, positive and negative; R. stolonifer, negative and positive; C. bertholletiae, equivocal and positive; and P. chrysogenum.
positive and negative. In pure cultures of C. albicans, F. solani, and S. apiospermum, results of both aspergillosis and mucormycosis IHC were negative.

**Accuracy of Histomorphologic Diagnosis of Mucormycosis and Aspergillosis**

We determined the accuracy of histomorphologic diagnosis of probable PM and probable IPA, using the IHC tests (Table 2). Of the 23 patients with probable PM, 1 had a positive fungal culture from sputum. However, 5 (31%) of 16 patients with probable PM whose GM assays were available had positive serum or BAL fluid GM results. Of the 23 with probable PM, 20 (87%) had positive mucormycosis IHC and 2 (9%) had positive aspergillosis IHC results (Figure 2A), including 1 with both. The other 2 patients (9%) had negative IHC results for both mucormycosis and aspergillosis.

Of the 16 patients with probable IPA, 5 (31%) had positive culture results from nonsterile specimens such as sputum or BAL fluid. Of the 16 with probable IPA in whom GM assays were available, 14 (88%) had positive serum or BAL fluid GM results; 10 (63%) had positive aspergillosis IHC results, 4 (25%) had positive mucormycosis IHC results (Figure 2B), and 2 (13%) were negative IHC results for both aspergillosis and mucormycosis. Of the 4 patients with probable IPA for whom mucormycosis IHC results were positive, none had positive fungal cultures, and all had positive serum or BAL GM results.

The agreement in histomorphologic diagnosis between the 2 experienced pathologists is shown in Table 3. The pathologists disagreed in 7 of the 54 cases, and the overall percentage agreement was 87% (κ = 0.75; excellent agreement).

**DISCUSSION**

In this study we found that the commercially available monoclonal antibodies for IHC were useful in overcoming the limitations of tissue morphology alone in distinguishing between mucormycosis and aspergillosis. In addition, when we evaluated the accuracy of histomorphologic diagnosis with IHC, about one-quarter of the probable IPA cases, based on tissue morphology alone with other mycologic evidence of aspergillosis, may have been PM mistaken for IPA, whereas most of the probable PM cases based on tissue morphology were probably diagnosed correctly.

Although direct comparisons are difficult because different antibodies were used in different studies, our findings are consistent with work showing that IHC could improve the sensitivity and specificity of diagnosis for systemic mycoses, especially aspergillosis [1, 7, 11, 12]. However, there is little information on the utility of mucormycosis IHC because of the few patients with mucormycosis studied previously. Our findings demonstrate that this test may be useful in the present clinical setting, where there is a low culture-positive rate of mucormycosis and no available biologic markers for mucormycosis. Without sterile culture results, definitive diagnosis of mucormycosis and aspergillosis is difficult. This assertion is supported by a previous study showing that only about two-thirds of histomorphologic aspergillosis (15 of 21) had positive aspergillosis IHC results [7]. Similarly, Lee et al [13] reported that only 43 of 52 patients (83%) with histomorphologic aspergillosis had positive cultures for Aspergillus species, and the remaining 9 (17%) had cultures positive for other organisms including Scedosporium and

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**Table 1. Diagnostic Performance of Mucormycosis and Aspergillosis Immunohistochemistry Tests in Proven Mucormycosis and Proven Aspergillosis**

<table>
<thead>
<tr>
<th>IHC Test Result</th>
<th>Proven Mucormycosis, No. of Cases (n = 7)</th>
<th>Proven Aspergillosis, No. of Cases (n = 8)</th>
<th>Diagnostic Performance % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mucormycosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>0</td>
<td>Sensitivity: 100 (65–100)</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>Specificity: 100 (65–100)</td>
</tr>
<tr>
<td><strong>Aspergillosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>7</td>
<td>Sensitivity: 87 (53–98)</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>1</td>
<td>Specificity: 100 (65–100)</td>
</tr>
</tbody>
</table>

**Abbreviations:** CI, confidence interval; IHC, immunohistochemistry.

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**Table 2. Accuracy of Histomorphologic Diagnosis in Patients With Probable Pulmonary Mucormycosis and Probable Invasive Pulmonary Aspergillosis**

<table>
<thead>
<tr>
<th>Results</th>
<th>Mucormycosis (n = 23)</th>
<th>Aspergillosis (n = 16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycologic evidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive culture</td>
<td>1 (4)a</td>
<td>5 (31)b</td>
<td>.03</td>
</tr>
<tr>
<td>GM assay</td>
<td>5/16 (31)</td>
<td>14 (88)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Serum</td>
<td>1/15 (7)</td>
<td>10/15 (67)</td>
<td>.001</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>4/8 (50)</td>
<td>9/11 (82)</td>
<td>.32</td>
</tr>
<tr>
<td>IHC results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucormycosis only</td>
<td>19 (83)</td>
<td>4 (25)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Aspergillosis only</td>
<td>1 (4)</td>
<td>10 (63)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Both positive</td>
<td>1 (4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Both negative</td>
<td>2 (9)</td>
<td>2 (13)</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** BAL, bronchoalveolar lavage; GM, galactomannan; IHC, immunohistochemistry.

a *Rhizopus* spp. were isolated from sputum culture.

b Two were isolated from bronchial aspirates culture, and 3 from sputum culture.
Fusarium spp. Thus, our findings on the limitations of tissue morphology alone for diagnosing aspergillosis are consistent with previous findings [1, 7, 11–13]. However, only limited data are available concerning the accuracy of histomorphologic diagnosis of mucormycosis. Jensen et al [7] showed that 1 of 2 mucormycosis cases had a positive aspergillosis IHC result. In the present work, we demonstrated with IHC testing that histomorphologic diagnosis of mucormycosis was more reliable than histomorphologic diagnosis of aspergillosis (Table 2).

The recent clinical trials and observational studies of invasive aspergillosis have used the EORTC/MSG criteria [10]. Hence, a large proportion of the patients with probable IPA enrolled in clinical studies that classify patients based on positive GM assays are regarded as having IPA [14]. However, our data clearly show that positive GM results are not uncommon in patients with proven mucormycosis or probable mucormycosis. These instances could be false-positives for the GM assay associated with use of antibiotics such as piperacillin-tazobactam, blood

Table 3. Agreement of Histomorphologic Diagnosis Between 2 Experienced Pathologists

<table>
<thead>
<tr>
<th>Diagnosis by Pathologist A</th>
<th>Diagnosis by Pathologist B, No. (%) of Patients</th>
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<tbody>
<tr>
<td></td>
<td>Morphologic Mucormycosis</td>
</tr>
<tr>
<td>Morphologic mucormycosis</td>
<td>23</td>
</tr>
<tr>
<td>Morphologic aspergillosis</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>23 (43)</td>
</tr>
</tbody>
</table>

* The overall percentage agreement was 87% (κ = 0.75).
products, or Plasma-Lyte [15–17]. In fact, all but 1 patient with PM whose GM assays were positive had received blood products or piperacillin-tazobactam, although the recent piperacillin-tazobactam preparations do not seem to cause false-positive GM results [18]. Another possible explanation could be cross-reaction between the mucormycosis antigen and GM. This hypothesis is supported by a report of positive BAL GM results in patients with invasive mucormycosis [19], although no cross-reactivity has been observed with culture supernatants of agents of mucormycosis [20, 21].

Alternatively, Aspergillus may colonize damaged bronchi in patients with mucormycosis. This colonization could be responsible for the positive GM assays because we have shown that the serum GM assay frequently has positive results in patients with simple aspergilloma without overt tissue invasion [17, 22]. Therefore, we assume that some of the cases classified as probable IPA in clinical studies may have been misdiagnosed and treated with voriconazole, with adverse outcomes. Interestingly, all 5 patients in clinical studies may have been misdiagnosed and treated with voriconazole, with adverse outcomes. Interestingly, all 5 patients with probable IPA and positive nonsterile culture results had positive aspergillosis IHC results only, compared with only 5 of 12 patients (42%) with probable IPA and positive GM assay results (Supplementary Table 3). We can infer from this data that morphologic diagnosis combined with nonsterile culture results is accurate, but morphologic diagnosis without culture results should be interpreted with caution.

Although newer methods such as IHC, fluorescent antibody staining [23], and in situ hybridization [24], may compensate for the limitations of morphologic diagnosis, histomorphologic diagnosis should always be confirmed by fungal culture. It is worth noting that, to increase the yield of fungal culture, microbiologists should chop rather than grind tissues when setting up mycologic cultures, because grinding gives a low culture rate for mucormycosis [25]. However, if a culture is not available or cannot be obtained, these newer methods may help obtain the most likely diagnosis possible. In addition, aware of the limitations of histomorphologic diagnosis, several authors have suggested that pathologists should describe the observed hyphae (septated vs paucisepptated, hyaline vs pigmented, plus morphologic features), followed by a comment indicating the various fungal genera that could have those morphologic features [26, 27].

This study has several limitations. First, in actual clinical practice most IPA cases are diagnosed and treated on the basis of radiologic findings and positive GM results without tissue samples being obtained. Hence, the true diagnosis in many patients with suspicious IPA who would have needed tissue biopsy is uncertain, and their cases could follow unusual clinical courses. Therefore, the fact that all the patients with probable IPA in this study underwent tissue biopsy may have created a kind of selection bias, which might partially explain the high proportion of positive mucormycosis IHC results in our patients. Second, we used IHC as the reference standard to determine the accuracy of histomorphologic diagnosis of probable cases. However, there were too few sterile culture-proven mucormycosis and aspergillosis cases to permit evaluation of the diagnostic performance of mucormycosis and aspergillosis IHC. To overcome the insufficient number of controls, we tested the cross-reactivity of the IHC with various fungal and nonfungal infections, and we demonstrated that there was no cross-reactivity of the IHC in fusariosis, scedosporiosis, candidiasis, or tuberculosis. We also evaluated the reactivity of aspergillosis IHC and mucormycosis IHC in pure cultures of various fungi.

Third, the sensitivity of Aspergillus IHC was suboptimal (Table 1), so it is difficult to draw a definite diagnosis in 2 of 16 patients (13%) with probable IPA with both negative aspergillosis and mucormycosis IHC results. However, 4 of 16 patients (25%) with probable IPA had positive mucormycosis IHC results only. Therefore, the suboptimal sensitivity of aspergillosis IHC does not alter the main finding on the limitation of tissue morphology in the diagnosis of aspergillosis. Fourth, some kind of week cross-reaction of mucormycosis IHC and aspergillosis IHC was noted in the pure cultures of A. fumigatus and C. bertholletiae. Of 23 patients with probable mucormycosis, 1 (4%) was positive for both aspergillosis and mucormycosis IHC. This dual-positive staining may be due to cross-reaction rather than coinfection, although one study has described multiple fungal infections involving concomitant pulmonary aspergillosis and mucormycosis [28]. In addition, when IHC tests show an Aspergillus-positive reaction and the fungus in tissue seems to be compatible with mucormycosis, it is difficult to draw a definite diagnosis. The development of more sensitive and specific fungal IHC tests will provide definite answers for this complex situation.

In conclusion, our data showed that tissue morphology alone is of limited use in the diagnosis of aspergillosis. In the absence of fungal culture results, the IHC tests seem to be helpful in differentiating between aspergillosis and mucormycosis.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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