Original Article

Comparison in Quantities from Including Angles Comprising Lines of Hypha Themselves in Histological Images between *Mucorales* and *Aspergillus*: An Exploration into Basic Algorithm Supporting Future Automated Histological Image Analyzing System to Isolate *Mucorales* from Other Filamentous Fungi

Naobumi Tochigi, Sota Sadamoto, Minoru Shinozaki, Megumi Wakayama and Kazutoshi Shibuya

Department of Surgical Pathology, Faculty of Medicine, Toho University

ABSTRACT

Background: The rate of aspergillosis has decreased due to improvements in therapy. The rate of mucormycosis, however, has gradually increased in recent years. Both aspergillosis and mucormycosis produce histologically similar hyphae, pointing to the need for an objective tool to distinguish between them. **Methods:** Three aspergillosis cases and three mucormycosis cases were selected from autopsy cases in our hospital. Representative histological images were captured and hyphal angles in extravascular and intravascular lesions were calculated. **Results:** For both extravascular and intravascular lesions, the average hyphal angle of aspergillosis was acute, and the standard deviation was less than that of mucormycosis. In aspergillosis, the average hyphal angle for extravascular lesions was acute, and the standard deviation was less than that for intravascular lesions. However, for mucormycosis, there was no significant difference in both the average and standard deviation of the hyphal angles. **Conclusion:** Surgical pathologists should carefully examine the histological characteristics of the fungus to correctly identify specimens and be able to administer proper therapies.

Key words : aspergillosis, hyphal angle, mucormycosis

Introduction

An effective drug for controlling aspergillosis was developed in the 1990s. The drug, however, exhibited little effect on mucormycosis. Recently, clinicians have recognized that mucormycosis occurring in agranulocytic individuals has a strong negative impact when they are immunocompromised. There is no definitive analytical tool for mucormycosis, such as beta-D-glucan or an antibody for a mucormycosis-specific antigen. In general, fungal cultures are specific, but are less sensitive and need a long time. In addition, fungi causing mucormycosis consist of multiple taxonomic clusters, and precise identification of species is difficult. Furthermore, in cases of invasive fungal infection, patients are often in a deteriorated state, due to, for example, hematopoietic malignancy.

Although there is limited capability for definitively identifying mucormycosis, the early stage can be detected through protocol computed tomography in febrile neutropenia. Some of these early-stage patients can recover via adequate treatment and surgical resection. Surgical pathologists, therefore, should make considerable efforts to extract information that could help identify the species based on histopathological specimens. It is also important to develop objective guidelines to avoid subjectivity among surgical pathologists. In this study, we examined the morphological difference between aspergillosis and mucormycosis, and found that their hyphal angles are different. Additionally, since it is important to analyze the difference between lesions, we observed both intravascular and extravascular lesions.

In the present study, we aimed to develop and evaluate an image-analyzing system to differentiate *Mucorales* from *Aspergillus* and other filamentous fungi using Grocott's

Address for correspondence: Naobumi Tochigi, MD, PhD

Department of Surgical Pathology, Faculty of Medicine, Toho University, 6-11-1, Omori-Nishi, Ota-ku, Tokyo, 143-8541 Japan

Received: 26, March 2019, Accepted: 6, September 2019

E-mail: naobumi.tochigi@med.toho-u.ac.jp

Fig. 1. GMS staining of aspergillosis cases. a and b; c and d; and e and f are from the same cases. a, c, and e are extravascular lesions, and b, d, f are intravascular lesions. Bar indicates 0.1 mm, and the magnification is $\times 20$.

methenamine silver (GMS) -stained formalin-fixed and paraffin-embedded (FFPE) sections. This system may serve as a basic algorithm for developing an automated image analysis system for the differential diagnosis of mycosis caused by Mucorales to enable accurate, rapid, and standardized diagnoses.

Materials and methods

This study was approved by the ethical committee of the Faculty of Medicine, Toho University (No. #A17123).

Patients

We reviewed our autopsy files and selected three aspergillosis and three mucormycosis cases. All the cases were acute myeloid leukemia cases with a status of post-systemic chemotherapy and a final peripheral leukocyte count of less than 1000/uL. The subjects of this study were all autopsy cases. In our hospital, all autopsies are performed with written permission that includes the consent for research.

Image analysis

We developed the software named “Check-Angle”. At first,

we captured representative images of both intravascular and extravascular lesions. Next, we put the start and finish of each fungus. After this procedure, this software automatically made the extension line. Repeating this procedure, this software also automatically measured the angle of each hypha, calculated the mean of all hyphae, and calculated the difference of the angle between the mean hyphae and each hypha. The minimal hyphal angle was 17 degrees, and the maximum was 91 degrees. For all cases, we examined and compared the intravascular and the extravascular lesions. If the difference from the mean angle was an obtuse angle, we calculated its supplement to get an acute angle, and used this angle for analysis.

Statistical analysis

We used the Mann-Whitney U test to evaluate the differences between angles and the F test to evaluate the differences between standard deviations.

Results

Each resulting angle was classified into one of four groups:

Fig. 2. GMS staining of mucormycosis cases. a and b; c and d; and e and f are from the same cases. a, c, and e are extravascular lesions, and b, d, f are intravascular lesions. Bar indicates 0.1 mm, and the magnification is $\times 20$.

Fig. 3. Index of analysis. Red line indicates extension of hyphal lines, and yellow angle indicates the measured angle. Bar indicates 0.1 mm, and the magnification is $\times 20$.

extravascular lesion of aspergillosis (AE), extravascular lesion of mucormycosis (ME), intravascular lesion of aspergillosis (AI), and intravascular lesion of mucormycosis (MI) (Fig. 1 and Fig. 2). We analyzed and compared the aspergillosis cases

and the mucormycosis cases (Fig. 3, Table 1). For both the extravascular and intravascular lesions, the average hyphal angle of aspergillosis was acute, and the standard deviation was less than that of mucormycosis. For aspergillosis, the average hyphal angle for extravascular lesions was acute, and the standard deviation was less than that of intravascular lesions. However, for mucormycosis, there was no significant difference in both the average and standard deviation of hyphal angles (Fig. 4).

Discussion

Invasive aspergillosis can now be controlled because of improvements in therapeutic drugs for hematopoietic malignancies, especially in supportive therapies. Recently, however, there has been an increase in frequency of mucormycosis, for which there are no positive analytical tools or effective drugs¹⁻³. It is very important to differentiate aspergillosis and mucormycosis because they have different therapeutic strategies. Aspergillosis and mucormycosis can likely be differentiated through improvements in standard clinical

Table 1. Analysis of each aspergillosis and mucormycosis cases. A1, A2, and A3 are aspergillosis cases; and M1, M2, and M3 are mucormycosis cases. The Mann-Whitney U test was used to evaluate the differences between angles and the F test to evaluate the differences between standard deviations.

Extravascular lesions			Intravascular lesions		
	t -test	F-test		t -test	F-test
A1-M1	$p < 0.001$	$p = 0.002$	A1-M1	$p < 0.001$	$p = 0.018$
A1-M2	$p < 0.001$	$p < 0.001$	A1-M2	$p < 0.001$	$p = 0.124$
A1-M3	$p < 0.003$	$p < 0.001$	A1-M3	$p = 0.051$	$p = 0.012$
A2-M1	$p < 0.001$	$p < 0.001$	A2-M1	$p = 0.288$	$p = 0.884$
A2-M2	$p < 0.001$	$p < 0.001$	A2-M2	$p < 0.001$	$p = 0.115$
A2-M3	$p < 0.001$	$p < 0.001$	A2-M3	$p = 0.612$	$p = 0.533$
A3-M1	$p = 0.047$	$p = 0.808$	A3-M1	$p = 0.043$	$p = 0.960$
A3-M2	$p = 0.107$	$p = 0.358$	A3-M2	$p < 0.001$	$p = 0.171$
A3-M3	$p = 0.255$	$p = 0.251$	A3-M3	$p = 0.968$	$p = 0.459$

Fig. 4. Distribution of four groups.

AE: extravascular lesion of aspergillosis, ME: extravascular lesion of mucormycosis, AI: intravascular lesion of aspergillosis, and MI: intravascular lesion of mucormycosis. Bar represents the mean plus or minus standard deviation. Error bar indicates 95% confidence interval.

microbiological approaches using sputum or bronchioalveolar lavage and staining behavior. However, especially for surgical specimens, degeneration may interfere with fungal growth, and typical morphology may not be observed. We previously reported *in situ* hybridization tools to identify and differentiate fungi⁴⁻⁶; however, not all laboratories use these tools. An improved method, therefore, is needed for use in general specimens.

Based on our experience, it is possible to differentiate between aspergillosis and mucormycosis using the hyphal angle of the fungus. To our knowledge, however, there is no research on the quantitative calculation of hyphal angles. In invasive fungal infection cases, fungi infect the lungs first,

then invade into vessels and cause sepsis. In our study, we focused on the difference between extravascular lesions and intravascular thrombi and found that hyphae in aspergillosis cases had stronger directional characteristics compared to mucormycosis cases. In addition, we found that there was no difference between extravascular lesions and intravascular lesions in mucormycosis cases. In contrast, for aspergillosis cases, the extravascular lesions had stronger directional characteristics than mucormycosis cases. In general, both extravascular and intravascular lesions can be observed in histological specimens. Careful examination of both extravascular and intravascular lesions from the same case may enable identification of the fungus in the specimen. In addition, the histological image analysis procedure described here can potentially serve as a basic algorithm for developing an automated image analysis system for the differential diagnosis of mucormycosis, enabling accurate, rapid, and standardized diagnoses.

Conflict of interest

K Shibuya has received research funding through personal speaker's fees from Sumitomo Dainippon Pharma Co., Ltd. and personal consultant fees from Miraca Holdings Inc. The other authors declare that they have no competing interests.

Author's contributions

NT performed the histological analysis and was a major contributor in writing the manuscript. MS prepared the histological specimens. SS, MW, and KS discussed the results. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Dr. Hisayuki Tsukuma for advice regarding statistical analysis, and Mr. Shinji Oura for developing the software to analyze the angles.

Sources of funding

This study was supported by AMED under Grant Number JP19fk0108045 and JP19fk0108094, JSPS KAKENHI Grant Number JP26860250, and Kurozumi Medical Foundation. The role of the funding body is in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

References

- 1) Kume H, Yamazaki T, Togano T, Abe M, Tanuma H, Kawana S, Okudaira M: Epidemiology of visceral mycoses in autopsy cases in Japan: comparison of the data from 1989, 1993, 1997, 2001, 2005 and 2007 in Annual of Pathological Autopsy Cases in Japan. *Med Mycol J* **52**: 117-127, 2011.
- 2) Shimodaira K, Okubo Y, Nakayama H, Katano H, Wakayama M, Shinozaki M, Ishiwatari T, Sasai D, Tochigi N, Nemoto T, Saji T, Kamei K, Shibuya K: Trends in the prevalence of invasive fungal infections from an analysis of annual records of autopsy cases of Toho University. *Mycoses* **55**: 434-443, 2012.
- 3) Suzuki Y, Kume H, Togano T, Ohto H: Epidemiology of Zygomycosis: Analysis of National Data from Pathological Autopsy Cases in Japan. *Med Mycol J* **58**: E89-E95, 2017.
- 4) Shinozaki M, Okubo Y, Sasai D, Nakayama H, Murayama SY, Ide T, Wakayama M, Hiruta N, Shibuya K: Identification of *Fusarium* species in formalin-fixed and paraffin-embedded sections by in situ hybridization using peptide nucleic acid probes. *J Clin Microbiol* **49**: 808-813, 2011.
- 5) Shinozaki M, Okubo Y, Sasai D, Nakayama H, Ishiwatari T, Murayama S, Tochigi N, Wakayama M, Nemoto T, Shibuya K: Development of a peptide nucleic acid probe to *Trichosporon* species and identification of trichosporonosis by use of in situ hybridization in formalin-fixed and paraffin-embedded (FFPE) sections. *J Clin Microbiol* **51**: 295-298, 2013.
- 6) Shinozaki M, Tochigi N, Sadamoto S, Yamagata Murayama S, Wakayama M, Nemoto T, Shibuya K: Technical Aspects and Applications for Developing in situ Hybridization Procedures for Formalin-Fixed and Paraffin-Embedded (FFPE) Tissues for Diagnosis of Fungal Infections. *Med Mycol J* **58**: E33-E37, 2017.