Using IFN-γ antibodies to identify the pathogens of fungal rhinosinusitis: A novel immunohistochemical approach

YUYAN YAN[1,2], ZUOTAO ZHAO[3], GEHONG DONG[1], YIDING HAN[1], DONGMEI YANG[1],
HONGYAN YIN[1], YINGSHI PIAO[1], CHUNYAN HE[1], CHENG TIAN[1], HONGFEI WAN[1],
XUE LI[1], YULAN JIN[1], JUGAO FANG[4] and HONGGANG LIU[1,2]

[1]Department of Pathology, Affiliated Beijing Tongren Hospital, Capital Medical University;
[2]Beijing Key Laboratory of Head and Neck Molecular Diagnostic Pathology, Beijing 100730;
[3]Department of Dermatology, First Hospital, Peking University, Beijing 100034; [4]Department of ENT,
Affiliated Beijing Tongren Hospital, Capital Medical University, Beijing 100730, P.R. China

Abstract. Fungal rhinosinusitis (FRS) is commonly caused by various *Aspergillus* species (spp) and Mucorales fungi, and the treatment and prognosis of cases differ depending on the causative fungus. The present study describes a novel immunohistochemical method that has high sensitivity and specificity for distinguishing between these two types of fungi in patients with FRS. Three groups were included in the study. Group A included formalin-fixed paraffin-embedded blocks of 51 nasal tissue specimens of patients with FRS (27 *Aspergillus* spp and 24 Mucorales) that were continuously obtained from the Department of Pathology of Tongren Hospital in Beijing as the experimental group and 34 cultures (26 *Aspergillus* spp and 8 Mucorales) of FRS that were randomly selected from the bacterial laboratory of Tongren Hospital in Beijing to verify the staining results of the paraffin-embedded blocks. Formalin-fixed paraffin-embedded blocks of 10 esophageal cancer specimens were included in Group B as the positive control group. All specimens in Groups A and B were stained with interferon-γ (IFN-γ) antibody. Group C consisted of the same specimens as described in Group A, however, when performing the immunohistochemical assay, IFN-γ antibody was replaced by PBS and this served as the negative control group. The differences in IFN-γ immunohistochemical staining between *Aspergillus* spp and Mucorales were analyzed. Staining of IFN-γ in paraffin-embedded samples was positive in 92.6% (25/27) of specimens in which *Aspergillus* spp were the causative pathogen, which was significantly higher compared with specimens in which Mucorales was causative (P<0.001), with only 4.2% (1/24) of specimens staining positive for IFN-γ. Immunohistochemical staining of cell cultures was 100% positive for *Aspergillus* spp, whereas all Mucorales were negative. Thus, the results of the current study indicated that IFN-γ antibody immunohistochemical staining may be used as a novel diagnostic tool to distinguish between *Aspergillus* spp and Mucorales when identifying the causative agent in FRS, providing a useful supplementary test to the current immunohistochemical methods in the clinical diagnosis of FRS.

Introduction

Fungal rhinosinusitis (FRS) is an infectious and/or allergic disease of the rhinosinuses caused by fungi. While considered uncommon, there have been an increasing number of cases reported over the last two decades (1). Invasive FRS (IFRS), a type of FRS, is an aggressive, often destructive and rapidly progressive infection, which is histopathologically characterized by the presence of hyphal invasion within the sinus mucosa, submucosa, blood vessels or bone and is classified as either acute or chronic (2). Acute invasive FRS (AIFRS), a subtype of IFRS, has a high mortality rate (50-80%) in immunocompromised patients (3). *Aspergillus* species (spp) and Mucorales are the major pathogenic fungi implicated in FRS. Different fungi have different pathogenic mechanisms and susceptibility to different antifungal drugs, thus cases of FRS that are caused by different types of fungi vary with regard to therapeutic options and the prognosis of patients. Mucorales infection exhibits a more rapid course of progression and greater invasiveness and is more likely to invade the arterial intimal layer, which can lead to thrombosis, hemorrhagic and ischemic necrosis and is, therefore, associated with high mortality rates (4,5). Currently, amphotericin B is the primary drug used to treat patients infected with Mucorales and intravenous voriconazole is the primary drug for *Aspergillus* spp infection. Accurate and timely diagnosis of the fungal species is of clinical importance; however, sensitive diagnostic assays have yet to be developed.

Correspondence to: Dr Honggang Liu, Department of Pathology, Affiliated Beijing Tongren Hospital, Capital Medical University, 1 Dongjiaominxiang, Dongcheng, Beijing 100730, P.R. China
E-mail: 18931107350ab@sina.com

At present, there are various laboratory tests that are used to identify fungi types, including culture, serological differentiation, molecular techniques and histopathological analysis (6). Because of its simplicity, the existence of established methods, reasonable cost and relatively fast and accurate diagnostic performance, histopathological analysis is one of the major methods applied to identify causative fungi clinically. Histopathological analysis includes the following staining techniques: Hematoxylin and eosin (H&E), Periodic Acid-Schiff (PAS), Gomori methenamine silver (GMS) and mucin 5B (MUC5B) immunohistochemical staining. Of the staining methods mentioned, MUC5B immunohistochemical staining has strong specificity and high sensitivity as it is based on an antigen-antibody response. However, not all *Aspergillus* spp cases are positively identified with MUC5B immunohistochemical staining. Additional immunohistochemical staining methods with strong specificity and high sensitivity are required to improve the histopathological diagnosis of FRS.

The present study aimed to develop a novel immunohistochemical staining assay to differentiate between *Aspergillus* spp and Mucorales, the two major types of pathogenic fungi implicated in FRS. Interferon-γ (IFN-γ) is a protein dimer that has an essential role in the innate and adaptive phases of an immune response (7,8). Although protein is a major component of the fungal cell wall, the presence of the IFN-γ antigen on the fungal cell wall has not yet been demonstrated. AIFRS animal pre-experiments revealed that *Aspergillus fumigatus* stained positive for IFN-γ. The hyphae and conidia of *A. fumigatus* were stained diffuse brown on the cell wall (Fig. 1). Therefore, it was hypothesized that the IFN-γ antigen may be specific to *Aspergillus* spp and that IFN-γ antibody may be used as a diagnostic marker to differentiate between *Aspergillus* spp and other major pathogenic fungi that cause FRS, including Mucorales. Validation of these assumptions may provide a novel method for identifying the causative fungal type in FRS with high specificity and sensitivity, which may allow earlier diagnosis and appropriate treatment of FRS. In the current study, an IFN-γ antibody immunohistochemical assay was performed on formalin-fixed paraffin-embedded nasal tissue specimens of FRS patients and H&E, PAS and GMS staining was performed for comparison, to observe the differences in the staining patterns of *Aspergillus* spp and Mucorales. In addition, IFN-γ antibody was used to stain cultures of *Aspergillus* spp and Mucorales, derived from FRS patients, to validate the results of the immunohistochemical assay on formalin-fixed paraffin-embedded specimens of FRS.

Materials and methods

Samples. Formalin-fixed paraffin-embedded blocks of 51 nasal tissue specimens of FRS patients (30 males and 21 females, mean age 32 years old; 27 infected by *Aspergillus* spp and 24 infected by Mucorales (*Rhizopus sp*.) were obtained from the Department of Pathology of Beijing Tongren Hospital. Samples were randomly selected based on a diagnosis of FRS from pathological and clinical data and fungal culture results between 2005-2012, which was when the FRS diagnosis was determined. Samples that were missing any of the aforementioned data were excluded. The cases in the present study included IFRS, fungal balls and allergic FRS. The types of causative fungi were blindly determined by two experienced pathologists following assessment of pathological sections stained with H&E, PAS and GMS, combined with clinical features and cultures, before immunohistochemistry assays were performed. The present study was approved by the ethics committee of the Beijing Tongren Hospital (Beijing, China).

Formalin-fixed paraffin-embedded blocks of 10 esophageal cancer specimens isolated from patients were randomly selected from the Department of Pathology of Beijing Tongren Hospital and blindly determined as such by two experienced pathologists. Cultures were provided by the bacterial laboratory of Tongren Hospital in Beijing. Randomly selected *Aspergillus* spp (*A. fumigatus*, n=10; *A. flavus*, n=8; and *A. terreus*, n=8) and Mucorales cultures (n=8) were cultured for 5 days at 25˚C on Vogel's glucose agar.

Groups. Group A was the experimental group and consisted of formalin-fixed paraffin-embedded blocks of 51 FRS specimens (27 *Aspergillus* spp and 24 Mucorales) and 34 cultures of these specimens (26 *Aspergillus* spp and 8 Mucorales). Group B consisted of formalin-fixed paraffin-embedded blocks of 10 esophageal cancer specimens, which served as the positive control group. Group C was the negative control group, in this group, IFN-γ antibody was replaced by PBS when performing immunohistochemical assay of Group A specimens.

Immunohistochemical assay of formalin-fixed paraffin-embedded specimens. Sections of formalin-fixed paraffin-embedded specimens (3 μm) were dried in an oven at 37˚C for 30 min, deparaffinized three times for 5 min in xylene and rehydrated with graded ethanol solutions and distilled water. Endogenous peroxidase in the sections was deactivated by incubating in 3% H_2O_2 for 10 min. Following rinsing with distilled water, antigen retrieval was performed using EDTA solution at a high temperature (800W) in a microwave oven for 8 min. After cooling in distilled water for 5 min and rinsing with PBS twice for 5 min, sections were subsequently incubated with primary antibody (IFN-γ antibody; orb10878; 1:200; Biorbyt Ltd., Cambridge, UK) overnight at 4˚C. The sections were subsequently rinsed with distilled water and PBS, and EnVision+ horseradish peroxidase rabbit antibody was added (K5007; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at room temperature (25˚C). Following washing with distilled water and PBS, the sections were incubated in 3,3'-diaminobenzidine solution (Dako; Agilent Technologies, Inc.) for 2-3 min. Chromogenic time was determined by observation using a light microscope. The sections were washed in distilled water, counterstained with hematoxylin and dehydrated with xylene prior to mounting. Esophageal cancer specimens were analyzed as positive controls using the same method. In negative controls, primary antibody was replaced by PBS.

Immunocytochemistry of cultures. *Mucor* cultures were added to slides, fixed in 95% ethanol for ~1 h at room temperature and incubated with the aforementioned IFN-γ antibody (1:200; Biorbyt) overnight at 4˚C. The remaining procedures were the same as those described in the previous

Table I. IFN-γ expression in *Aspergillus* spp and Mucorales formalin-fixed paraffin-embedded specimens.

	IFN-γ expression	
Fungal type	Positive cases (%)	Negative cases (%)
Aspergillus spp[a]	25 (92.5)	2 (7.4)
Mucorales[b]	1 (4.2)	23 (95.8)

[a]P<0.001 vs. *Aspergillus* spp group; [b]P<0.001 vs. *Mucor* group. Total cases, n=51; *Aspergillus* spp cases, n=27; Mucorales cases, n=24. IFN-γ, interferon-γ; spp, species.

Figure 1. IFN-γ immunostaining of *Aspergillus fumigatus* in an acute invasive fungal rhinosinusitis rat model in the pre-experiment. The cell walls of the hyphae are positively stained for IFN-γ (x40 magnification). IFN-γ, interferon-γ.

section for the immunohistochemical assay of formalin-fixed paraffin-embedded specimens. Esophageal cancer specimens were used as positive controls. PBS was used instead of primary antibody in the negative controls. The immunohistochemical processes described were also repeated with another IFN-γ antibody (PTG:15365-1-AP; 1:100; Proteintech Group, Inc., Chicago, IL, USA) to verify the results.

Statistical analysis. Statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The significance of the results was evaluated by the χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

The majority of *Aspergillus* spp have segmented forms and a shape that resemble antlers. Mucorales species are thicker and the majority have right angled hyphae. However, in certain clinical cases, *Aspergillus* spp become thicker and deformed, perhaps affected by the clinical characteristics of patients or the process of preparing sections, making it difficult to distinguish them from other fungi based on simple morphology. Thus, a highly specific immunohistochemical method to identify fungal types is required. The standard for a positive immunohistochemistry result in the present study was a brown staining of the cell wall or cytoplasm of the fungus.

Immunohistochemistry of formalin-fixed paraffin-embedded specimens. The results of immunohistochemical staining with IFN-γ antibody in formalin-fixed paraffin-embedded FRS specimens are presented in Table I and Fig. 2. IFN-γ expression was positive in 25 and negative in 2 *Aspergillus* spp specimens. Therefore, the IFN-γ-positive rate in *Aspergillus* spp was 92.6% (25/27). The hyphae and conidia of *Aspergillus* spp exhibited diffuse brown staining on the cell wall (Figs. 3, 4 and 5). The two negative *Aspergillus* cases were fungal balls. *Aspergillus* spp specimens from cases with allergic FRS and IFRS were all stained positive. By contrast, IFN-γ expression was negative in all Mucorales cell walls (Fig. 6), with the exception of one case, therefore, IFN-γ staining was positive in only 4.2% of Mucorales specimens; this was significantly lower than the percentage of *Aspergillus* spp specimens that stained positive (P<0.001).
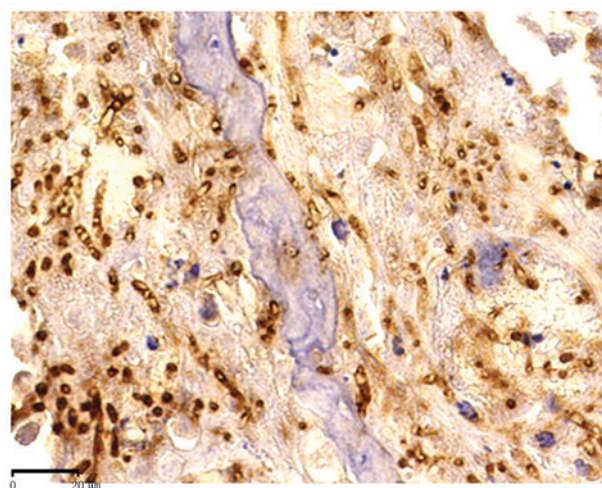

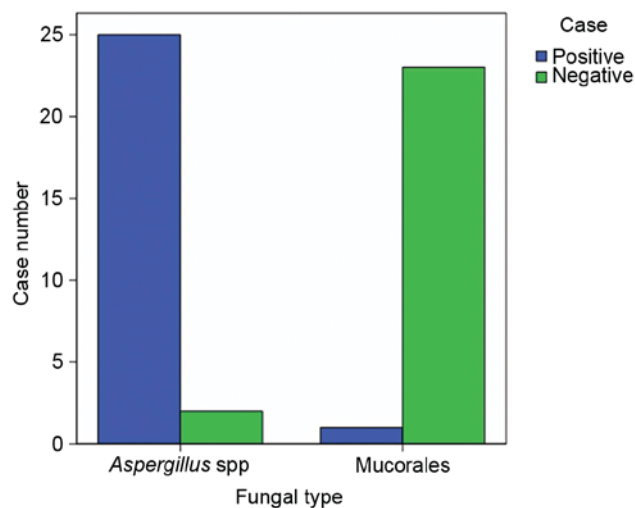Figure 2. Number of cases that exhibited positive and negative interferon-γ expression in *Aspergillus* spp and Mucorales formalin-fixed paraffin-embedded specimens.

Immunocytochemistry of cultures. The results of immunocytochemical staining of cultures for IFN-γ are summarized in Table II, which demonstrates that positive immunoreactivity was observed in all *A. fumigatus* (Fig. 7), *A. flavus* (Fig. 8) and *A. terreus* (Fig. 9) cultures. The positive staining rate for *Aspergillus* spp was 100% (26/26), whereas all *Mucor* spp (Fig. 10) stained negative for IFN-γ. The cell walls of positive hyphae and conidia exhibited diffuse brown staining. A significant difference in IFN-γ expression was observed between *Aspergillus* spp and *Mucor* spp (P<0.001). In the positive control group (Group B), all cancer cell membranes exhibited positive brown staining, while all specimens in the negative control group (Group C) exhibited negative staining results. Identical immunohistochemical staining results from the paraffin-embedded sections and the cultures were obtained with the two IFN-γ antibodies from different manufacturers.

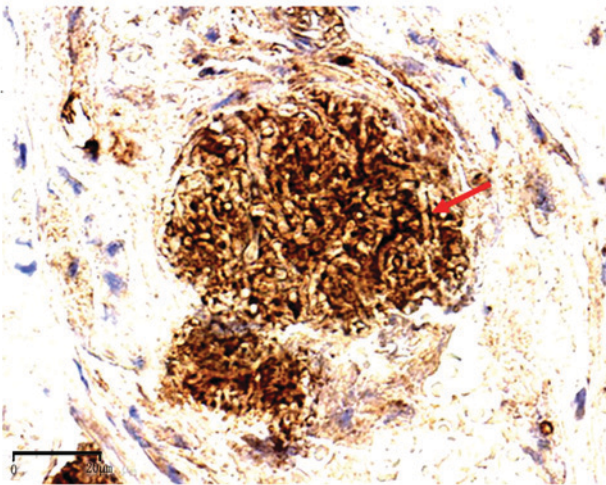Figure 3. IFN-γ immunostaining of *Aspergillus flavus* invading the vessels of human rhinosinuses. The cell walls of the hyphae are positively stained for IFN-γ (x40 magnification). IFN-γ, interferon-γ.
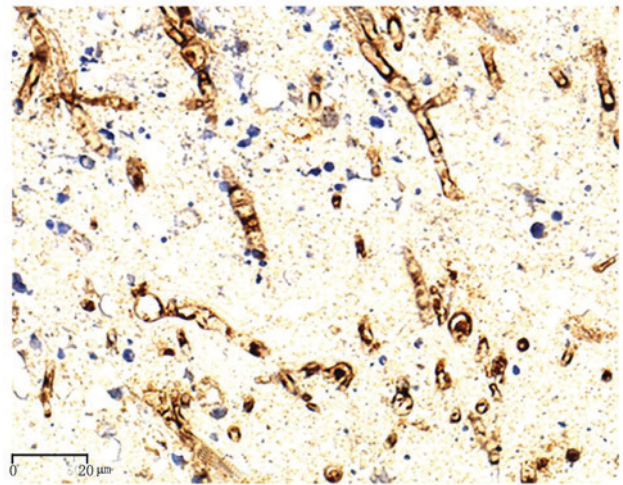

Figure 5. IFN-γ immunostaining of *Aspergillus fumigatus* in allergic fungal rinosinusitis. The cell walls of the hyphae are positively stained for IFN-γ (x40 magnification). IFN-γ, interferon-γ.
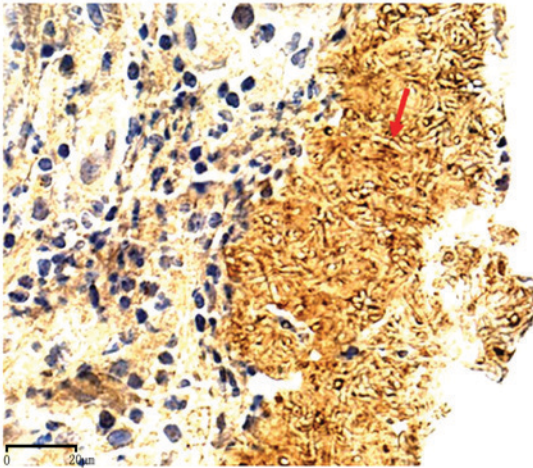

Figure 4. IFN-γ immunostaining of *Aspergillus fumigatus* in a fungal ball of rhinosinus. The cell walls of the hyphae are positively for IFN-γ. x40 magnification. IFN-γ, interferon-γ.

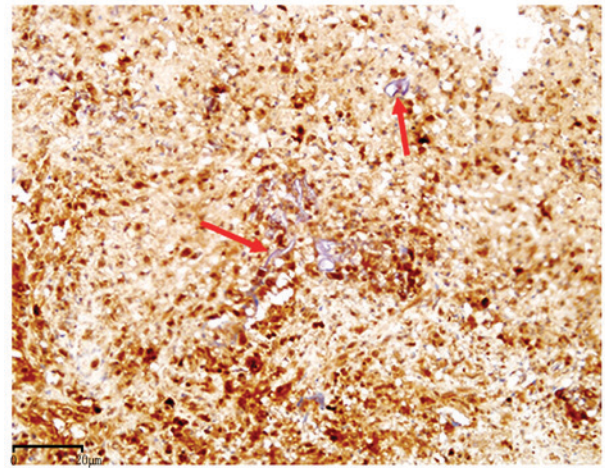

Figure 6. IFN-γ immunostaining of *Rhizopus* (a common genus of Mucorales) in invasive fungal rhinosinusitis, which was negative for IFN-γ (x40 magnification). IFN-γ, interferon-γ.

Discussion

The present study performed immunohistochemical staining for IFN-γ in samples of FRS and may have developed a novel diagnostic immunohistochemical approach to distinguish between types of fungi that are associated with FRS, which may increase the potential for earlier and more accurate diagnosis and treatment of FRS.

FRS was once considered to be a rare disorder; however, it is currently reported with increasing frequency worldwide (9-11). In the current classification system, FRS is classified into invasive and noninvasive types (12). Based on guidelines from the International Society for Human and Mycology Group, IFRS may be classified as acute, chronic or granulomatous, while the non-invasive types of FRS are allergic FRS and fungus balls (13). IFRS is a lethal disease that has a fatality rate of 50-80% in AIFRS (3). Currently, surgery and antifungal drugs are the major treatments for FRS. Early, accurate diagnosis and systematic antifungal

drug treatment is critical for the management of FRS. *Aspergillus* spp and Mucorales are the major types of pathogenic fungi that cause FRS. *Aspergillus* spp account for 80% of FRS cases, while Mucorales are the most aggressive and dangerous pathogens that have been implicated in FRS, which means they are associated with a high mortality rate (3,14,15). The pathogenic mechanism of different fungi can vary and they may also be susceptible to different antifungal drugs. Therefore, a method that can rapidly and accurately identify the types of fungi that are causative in different FRS cases is key for early diagnosis and the appropriate treatment of FRS.

In recent years, various methods have been developed to identify the types of fungi that are implicated in FRS cases. Culture and histopathology-based tests have been supplemented with molecular and proteomic techniques, and also antigen detection methods (16-18). However, all methods possess limitations. The culture method is insensitive, has potential for laboratory contamination and often takes too

Table II. IFN-γ expression in of *Aspergillus* spp and *Mucor* spp cultures.

Fungal type	IFN-γ expression	
	Positive cases (%)	Negative cases (%)
Aspergillus spp[a]	26 (100)	0 (0)
Mucor[b]	0 (0)	8 (100)

[a]P<0.001 vs. *Aspergillus* spp group; [b]P<0.001 vs. *Mucor* group. Total cases, n=34; *Aspergillus* spp cases, n=26; *Mucor* cases, n=8. IFN-γ, interferon-γ; spp, species.

Figure 9. IFN-γ immunostaining of *Aspergillus terreus* cultures. The cell walls of hyphae and conidia are positively stained for IFN-γ (x100 magnification). IFN-γ, interferon-γ.
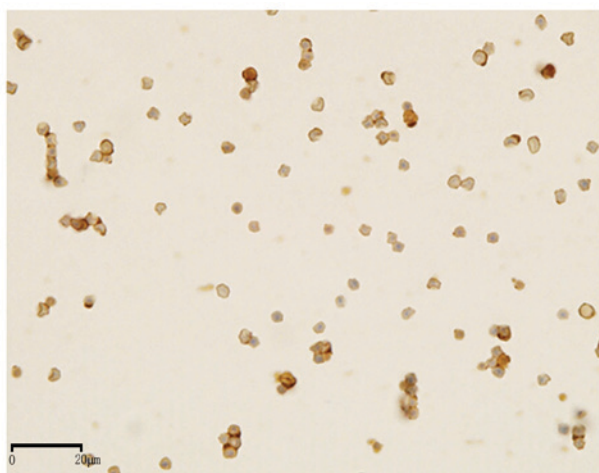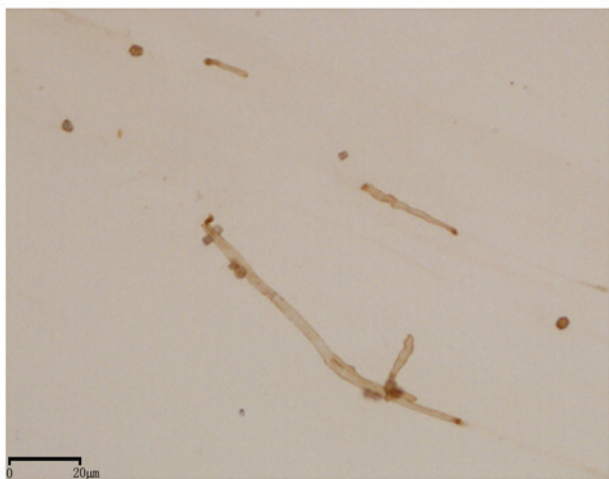

Figure 7. IFN-γ immunostaining of *Aspergillus fumigatus* cultures. The cell walls of conidia are positively stained for IFN-γ (x100 magnification). IFN-γ, interferon-γ.

Figure 10. IFN-γ immunostaining of *Mucor* spp cultures, which stained negative for IFN-γ (x40 magnification). IFN-γ, interferon-γ.

Figure 8. IFN-γ immunostaining of *Aspergillus flavus* cultures. The cell walls of hyphae and conidia are positively stained for IFN-γ (x40 magnification). IFN-γ, interferon-γ.

long (usually 5 to 12 days) to obtain results. Although molecular assays, including nucleic acid amplification tests and nucleic acid hybridization, have potential as they exhibit high sensitivity and spec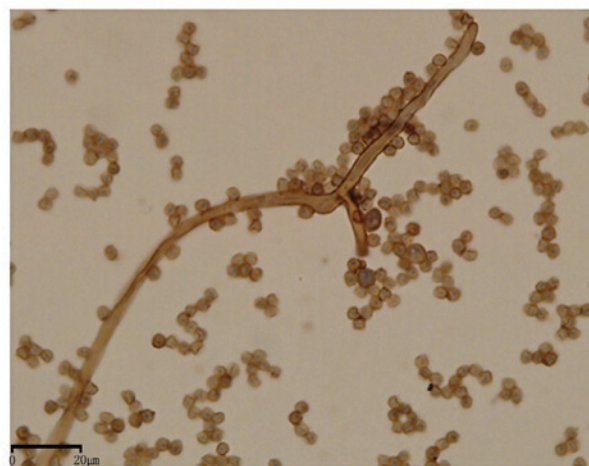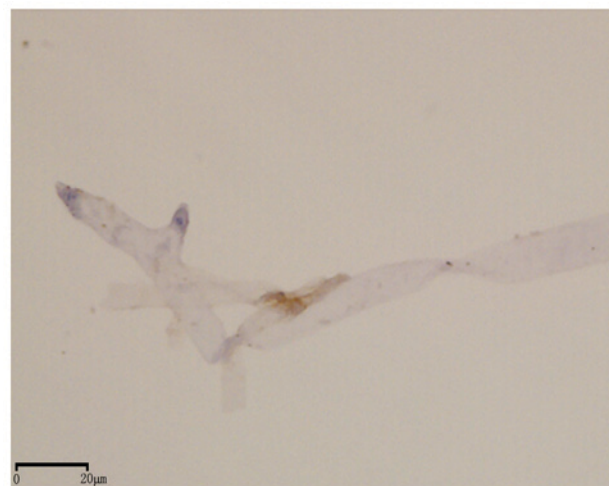ificity, the lack of test standardization and limited validation data for many fungal nucleic acid tests have hindered their general acceptance and broad application in clinical laboratories (16). The disadvantages of protein pattern recognition, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry, include the requirement of culture (they are not amenable to direct sample testing), database supplementation (particularly for molds) and, occasionally, manual spectral analysis (19). The application of the antigen detection method in immunocompromised patients is limited. Although histopathology-based testing also possesses limitations, it has many advantages in clinical application, including the fact that pathologists are familiar with it, it is easy to perform, reagents are readily available, it provides rapid diagnosis and has relatively high accuracy. Therefore, at present, histopathological identification is the definitive method for diagnosing fungal sinusitis in clinics (20).

IFN-γ is a protein dimer that has an essential role in the innate and adaptive phases of an immune response (8,21,22). It is required for optimal activation of phagocytes, it collaborates

in the generation of the protective antibody response and favors the development of a T helper 1 cell protective response (7,8). In addition to polysaccharides, protein is also an important component of the fungal cell wall. However, prior to the present study, it was not established whether the fungal cell wall contained IFN-γ antigen. In a pre-experiment on AIFRS animals, *A. fumigatus* in all infected cases exhibited brown staining when IFN-γ antibody was applied. Therefore, it was hypothesized that IFN-γ may be one of the components of the *Aspergillus* cell wall and IFN-γ antibody may be used to identify the causative fungal types in cases of FRS.

The results of the present study demonstrated that all *Aspergillus* spp and Mucorales specimens exhibited positive staining with H&E, PAS and GMS. GMS exhibited the strongest staining contrast between *Aspergillus* spp and Mucorales (data not shown). However, none of the tests accurately identified the fungal type, particularly for deformed and swollen fungi. Based on the principle of the specific antigen-antibody reaction, immunohistochemical staining with IFN-γ antibody was markedly different between *Aspergillus* spp and Mucorales, 92.5% *Aspergillus* spp stained positive, with diffuse brown staining on the cell wall, whereas Mucorales cell walls were all negatively stained, with the exception of one case. These results demonstrated that this may be a potentially useful, differential diagnostic test to distinguish between *Aspergillus* spp and Mucorales fungi.

In order to confirm that the IFN-γ antigen was of fungal origin and not from the host tissue, the present study also performed immunocytochemistry on cultures of *Aspergillus* spp and Mucorales using IFN-γ antibody. Again, IFN-γ expression was observed in the cell walls of all *Aspergillus* spp, including *A. fumigatus*, *A. flavus* and *A. terreus*. By contrast, all Mucorales cell walls were negative. These results confirmed that IFN-γ antibody may be used as a new supplementary test for the clinical diagnosis of FRS.

In order to verify that it was the IFN-γ antibody, and not other substances present in the primary antibody, that had positively stained the *Aspergillus* spp, the present study applied a second IFN-γ antibody produced by a different manufacturer to repeat the staining process and the observed results were identical, which further confirmed the reliability of this staining method for identifying fungal pathogens in FRS. In conclusion, immunohistochemical staining with IFN-γ is a novel method for distinguishing between *Aspergillus* spp and Mucorales, and may be a beneficial supplementary test for current immunohistochemical methods in the diagnosis of FRS. IFN-γ may be one of the antigen components of the cell wall of *Aspergillus* spp, however, this requires further studies.

Acknowledgements

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