Molecular Diagnosis of Cerebral Aspergillosis by Sequence Analysis With Panfungal Polymerase Chain Reaction

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Abstract: The authors describe a patient with osteosarcoma in whom a brain abscess developed after autologous peripheral stem cell transplantation. Serologic markers of fungal infection were negative, but fungal DNA was detected in the cerebrospinal fluid (CSF) by panfungal polymerase chain reaction (PCR) assay using primers derived from fungal 18S ribosomal RNA (rRNA) genes. The sequence of PCR products on the panfungal assay was identical to the 18S rRNA genes of *Aspergillus* species. The combination of sequence analysis and panfungal PCR assay could be useful in the diagnosis of cerebral aspergillosis.

Key Words: brain abscess, central nervous system, osteosarcoma, polymerase chain reaction, ribosomal RNA

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Fungal brain abscess is a life-threatening complication with a high mortality rate in immunocompromised patients, including stem cell or liver transplantation recipients.[1,2] Although early introduction of antifungal therapy is related to a favorable outcome,[1,3] the precise diagnosis of central nervous system (CNS) fungal infection is not easily established. Conventional diagnostic kits, which can detect fungal antigen or antibodies, and blood culture are inadequate for early diagnosis because of their lack of high sensitivity or specificity.[4,5] To circumvent these problems, a polymerase chain reaction (PCR) system of detecting fungal DNA has been developed recently, and because *Candida* and *Aspergillus* species are responsible for most of the invasive fungal infections in immunocompromised patients, several protocols for the detection of the DNA of these fungi with specific primers have been established.[6,7] Moreover, because the incidence of infections with uncommon fungal species has increased, panfungal PCR assays, which can detect a broad range of fungal pathogens, have also been developed.[8,9] However, identification of the pathogenic fungal species is not easy even if the panfungal PCR primers detect fungal DNA. Species-specific DNA probes following the panfungal PCR assay have been used to identify the pathogenic species,[10] but it is still not well established that these methods are adequate for clinical cases. This study evaluated whether sequence analysis following panfungal PCR assay is useful for the diagnosis of CNS fungal infection and identification of the pathogen.

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PATIENTS AND METHODS

Patient

A 15-year-old girl underwent autologous peripheral stem cell transplantation (PSCT) for osteosarcoma that had been diagnosed 2 years earlier. The conditioning regimen was etoposide, carboplatin, and ifosfamide. A high fever developed 5 days after PSCT, but physical examination of her chest, heart, and abdomen showed no abnormalities, nor were there signs of meningitis. A chest x-ray revealed no abnormalities. Treatment with panipenem (carbapenem antibiotics), vancomycin, and high-dose fluconazole (400 mg/day) was initiated, but the high fever continued and serum levels of C-reactive protein increased markedly (165 mg/L, normal levels <3 mg/L). She received granulocyte transfusion therapy 7 days after the PSCT, and the high fever rapidly subsided. Because a high fever did not develop again and the neutrophil count had exceeded 1.0×10^9/L 10 days after transplantation, the administration of high-dose fluconazole was discontinued on day 13. However, she complained of headache and weakness in the left hand and leg on day 21, and a neurologic examination revealed hemiparesis. A magnetic resonance imaging (MRI) scan of the brain showed multiple lesions with ring enhancement, consistent with a cerebral abscess (Fig. 1A). She had no history of fungal infection. Cerebrospinal fluid (CSF) examination showed normal values for cell count, protein, and glucose. Neither bacteria nor fungus was cultured from urine, respiratory secretions, blood, or CSF during the clinical course. The levels of (1→3)-β-D-glucan in plasma were normal continuously. Examination of the CSF sample was also normal (on days 55 and 69). Antigens of *Candida*, *Aspergillus*, and *Cryptococcus* were undetectable in blood or CSF, and antibodies against these fungi were also negative in both blood

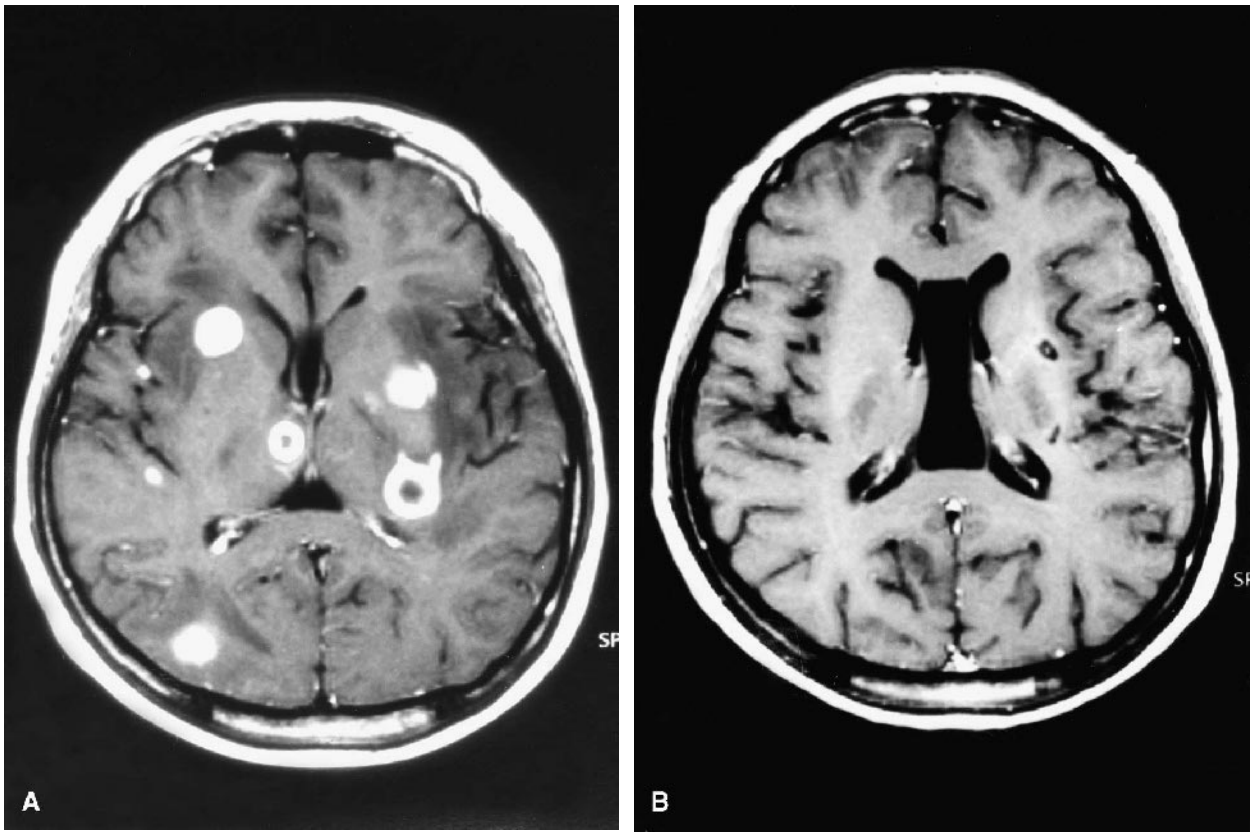

FIGURE 1. A, Axial MRI of the brain enhanced with gadolinium shows ring-enhancing lesions in both basal ganglia. B, Axial MRI of the brain enhanced with gadolinium shows small multiple lesions with ring enhancement in the left basal ganglia 6 months after the peripheral stem cell transplantation.

and CSF samples. Parents are often reluctant to agree with proceeding to brain biopsy. High-dose fluconazole (400 mg/day) was administered again. Clinical signs and symptoms improved, and serial MRI scans showed steady regression of the brain abscess. The neurologic examination was normal 6 months after PSCT, but a cerebral MRI showed small multiple lesions with ring enhancement despite continuation of treatment with fluconazole (see Fig. 1B).

Methods

A CSF sample was obtained under sterile conditions, and fungal DNA was detected by the method originally described by Makimura et al.[8] Panfungal PCR was carried out with universal primers (forward primer B2F [5′-ACTTTCGATGGTAGGATAG-3′] and reverse primer B4R [5′-TGATCGTCTTCGATCCCCTA-3′]), the sequences of which were derived from a consensus sequence of the 18S ribosomal RNA (rRNA) genes of various fungal pathogens, including *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*, and *Pneumocystis carinii*. The PCR products were transferred onto nylon membranes by Southern blot technique, followed by hybridization with a panfungal probe 18SIN3 (5′-CTGAGAAACGGCTACCACAT-3′). The CSF sample, which was positive for fungal DNA by the aforementioned panfungal PCR, was used for sequence analysis. Nested primer sets were constructed to amplify the 18S rRNA genes of medically important fungi. B2F and B4R were used as the primer pair in the first-round PCR and 18SIN3 was the forward primer in the second round. Conserved regions of the 18s rRNA gene for the second-round reverse primer were identified by aligning 16 organisms: *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondi*, *C. glabrata*, *C. krusei*, *C. kefyr* and *C. lusitaniae*, *Hansenula polymorpha*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *P. carinii*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Blastomyces dermatitis,* and *Coccidioides immitis* (GenBank accession numbers M60302, M60308, M60307, M60304, M60311, M60305, M60303, M60306, M60310, V01335, M55625, X12708, M55626, M55628, M55624, and M55627). The sequence of the reverse primer in the second round was as follows: AT18S 5′-YTGAATACTGATGCCCCCGA-3′. The locations of the primer pairs are shown in Figure 2. The first round of PCR amplification was 30 cycles as follows: at 94°C for 1 minute, at 55°C for 2 minutes, and at 72°C for 3 minutes with a terminal

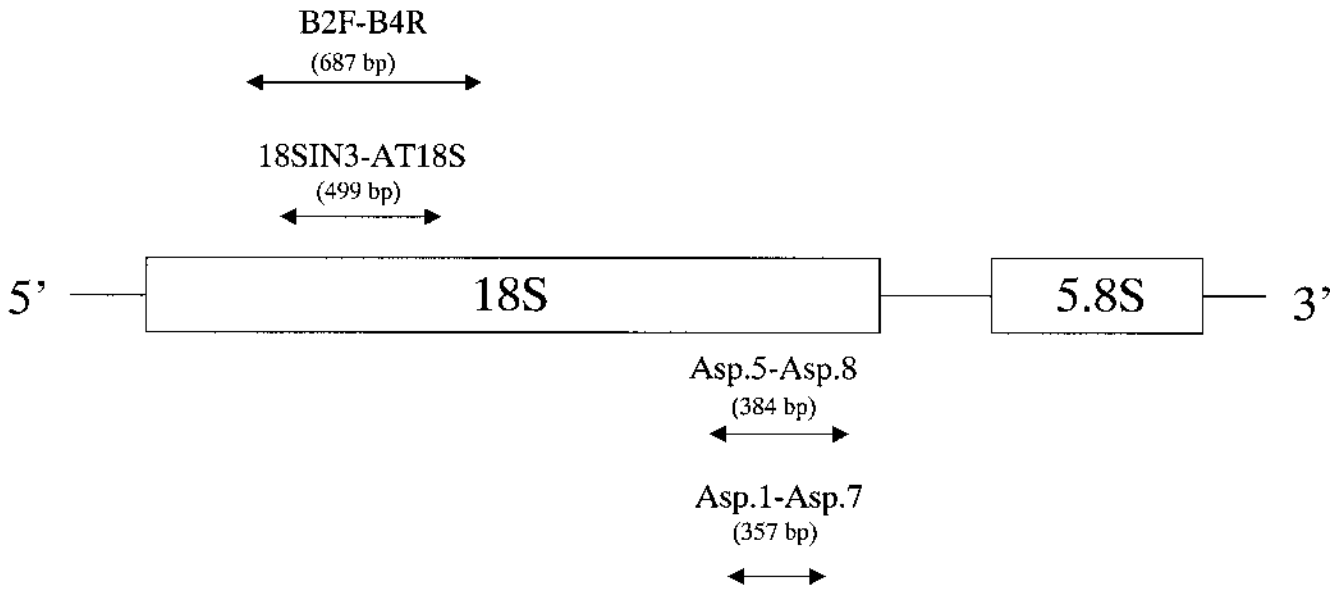

FIGURE 2. Locations of the primers used for PCR. The sequence of the primers was derived from the fungal 18S rRNA genes.[6] Asp.5-8 and Asp.1-7 are *Aspergillus*-specific primer pairs.[5]

step of 10 minutes at 72°C. Then, 2 μL of the first PCR product was reamplified with the inner primers for 30 cycles as follows: at 94°C for 1 minute, at 60°C for 2 minutes, and at 72°C for 3 minutes with a terminal step of 10 minutes at 72°C. The products of the expected size (499 bp), with a few base variations, among the different fungi were visualized under ultraviolet light. To prevent contamination, DNA extraction was always performed under filtered air, and negative controls were included during each DNA extraction procedure and in each step of the PCR. Cloning and sequencing of the fungal DNA were performed using a cloning kit (Original TA Cloning Kit; Invitrogen, Carlsbad, CA).

RESULTS

Fungal DNA was detected by panfungal PCR in a CSF sample obtained 55 days after PSCT. To identify the pathogen, the CSF sample was used for sequencing and four clones were isolated, with sequences identical to the 18S rRNA genes of *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. oryzae, A. tamarri*, *A. parasiticus,* and *A. sojae*) (Fig. 3). To confirm the CNS infection pathogen as *Aspergillus*, an *Aspergillus*-specific PCR assay was performed as described by Yamakami et al[7] (see Fig. 2). *Aspergillus* species DNA was detected in the CSF samples (day 55 and 69).

DISCUSSION

The diagnosis of CNS fungal infection can be confirmed by brain biopsy, but an invasive diagnostic procedure is not always feasible for immunocompromised patients. In the case we report here, panfungal PCR and sequencing revealed the pathogen of a brain abscess despite no other fungal infection markers. Although PCR products were cloned and sequenced in this case, we got the same results using direct sequencing (data not shown) of PCR products. The precise and quick diagnosis of fungal infection could be useful in selecting antifungal therapies.

Although a CSF sample from the patient was positive using a panfungal PCR assay, all other serologic markers of fungal infection were negative. There are two possible explanations for these findings. First, the levels of fungi in both blood and CSF were below the lower limit of detection of conventional diagnostic assays. Second, the CNS was infected with an uncommon fungal pathogen that could not be detected by conventional diagnostic assays. This study showed that the sequence of the panfungal PCR products was identical to the 18S rRNA genes of *Aspergillus* species, which have been reported to be the most prevalent fungi (30–50%) in fungal brain abscess following bone marrow transplantation.[1] Therefore, no serologic markers of fungal infection were due to the lack of sensitivity of conventional diagnostic assays. To confirm the CNS infection with *Aspergillus*, PCR was performed using *Aspergillus* species-specific primers. The sequence analysis following panfungal PCR assay showed that the fungal DNA was identical to that of six species of *Aspergillus* (*A. fumigatus*, *A. flavus*, *A. tamarii*, *A. sojae*, *A. parasiticus*, and *A. oryzae*). Among the *Aspergillus* species, *A. fumigatus* is the most frequent and important pathogen, but *A. flavus*, *A. oryzae*, *A. tamarii*, and *A. restrictus* have been reported as human pathogens.[1,11–13] To identify the precise species, an additional sequence analysis using *Aspergillus* species-specific genes

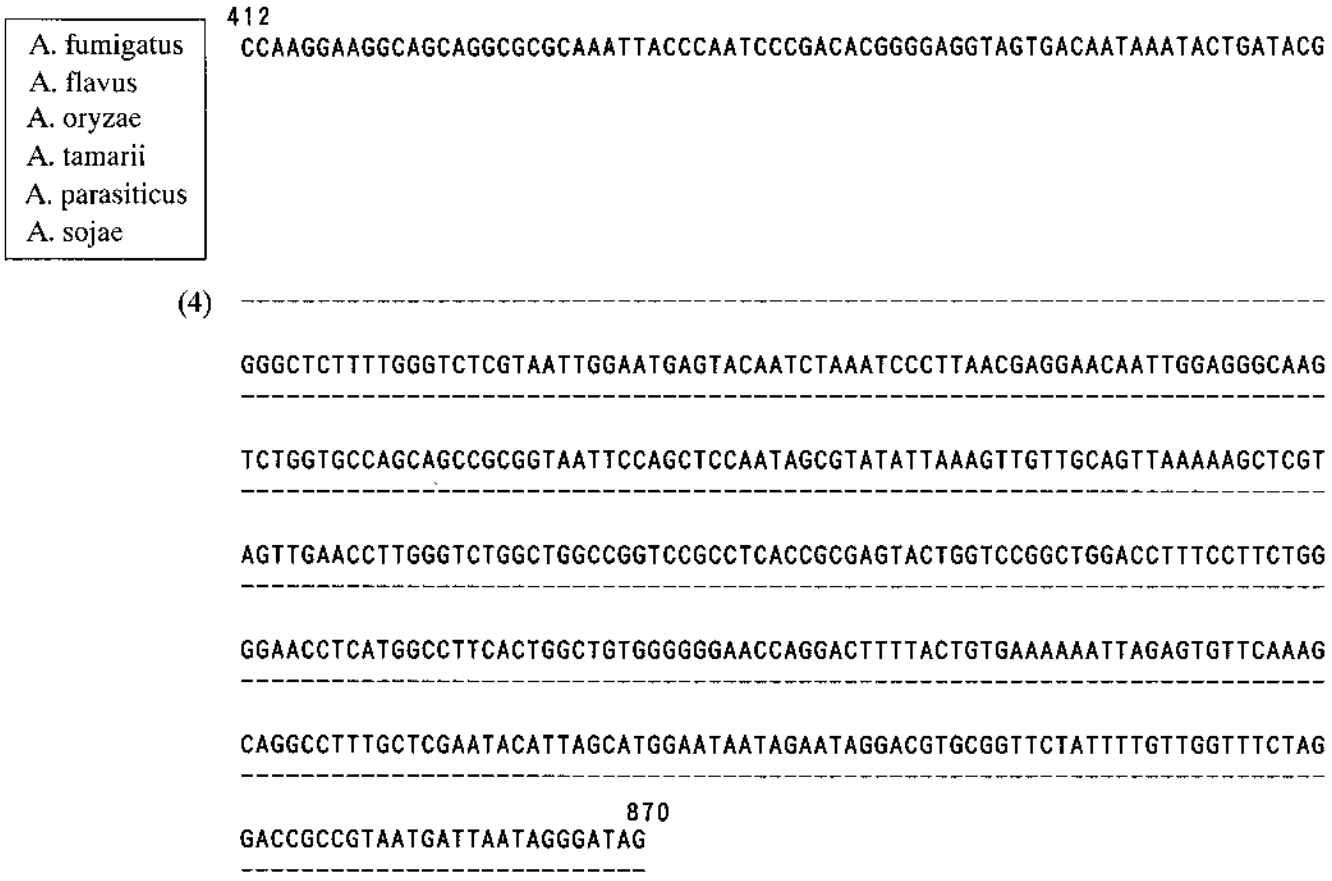

FIGURE 3. Nucleotide sequence of a PCR product using primer pairs B2F-B4R and 18SIN-AT18S. The number of clones sequenced is shown in parentheses. The sequences of the four clones were identical to the 18S rRNA genes of *A. fumigatus, A. flavus, A. oryzae, A. tamarri, A. sojae,* and *A. parasiticus*. Nucleotide number according to the 18S rRNA genes of *A. fumigatus* (M55626).

would be needed. Recently, Kami et al[14] reported that both a latex agglutination test and enzyme-linked immunosorbent assay were negative for a CSF sample in a patient with cerebral aspergillosis, but that *Aspergillus* DNA was detected in the CSF by nested PCR. The failure to detect the galactomannan antigen in the CSF was probably associated with the difference in the lower limit of detection between the conventional diagnostic tests and the PCR assays.[15]

Although the majority of patients with an *Aspergillus* brain abscess have pulmonary involvement,[1,16] our patient did not have any signs or symptoms of lung disease and no abnormalities were seen on the chest x-ray; the computed tomography scan of the chest was also normal. Several studies reported that the CNS lesion is the primary site of *Aspergillus* infection.[16–20] In this patient, there was a possibility that an intravenous catheter was the source of *Aspergillus* infection, but microscopic examination and culture of the catheter were negative.

Amphotericin B is recommended for the treatment of invasive aspergillosis, whereas fluconazole is not effective against invasive aspergillosis. However, it is difficult to achieve effective amphotericin B levels in CSF. Because our patient had mild renal dysfunction at that time, we did not administer amphotericin B. The patient responded to high-dose fluconazole, but CNS lesions did not disappear completely. This observation strongly suggests that conventional or liposomal amphotericin B is the gold standard for the treatment of cerebral aspergillosis. In summary, this study shows that the combination of sequence analysis with panfungal PCR assay is useful for the diagnosis of cerebral aspergillosis, especially in immunocompromised patients without any circulating fungal infection markers.

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