Analysis by Pulsed-Field Gel Electrophoresis of Candida albicans that Developed Resistance during Antifungal Therapy

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Abstract

A patient with myelofibrosis complicated by recurrent candidemia died despite treatment with amphotericin B and fluconazole. Autopsy revealed systemic candidiasis with fungal verrucae in the right ventricle and the root of the pulmonary artery. The strains of Candida albicans isolated from the blood had become resistant to amphotericin B and fluconazole during therapy, as well as to otherazole antifungals that had not been used. Pulsed-field gel electrophoresis showed that the resistant isolates had the same genotype as the sensitive strains isolated before treatment, but a chromosomal change in >2.0 Mb-bands was observed after treatment. It was thus proved that these repeatedly isolated C. albicans strains which were causing the continued fungemia in our patient were all the same strain and were acquiring resistance to antifungal agents during the therapy.

Key words: genotyping Candida albicans, antifungal agents, drug-resistant, myelofibrosis

Introduction

Recent progress in the treatment of various malignant diseases has enabled immunocompromised patients to survive considerably longer. However, there is a continuing increase worldwide in the number of patients with systemic fungal infections, particularly patients with complicated hematologic malignancies. Such individuals usually succumb within a short time unless effective antifungal chemotherapy and concomitant treatment of the underlying disease is prescribed. Systemic mycosis, including candidiasis, cryptococcosis, and aspergillosis, are being diagnosed with increasing frequency in acquired immunodeficiency syndrome (AIDS) patients.1,2) Few comparative data on the susceptibility of strains isolated before, during, and after treatment are available.

This paper describes the data obtained by pulsed-field gel electrophoresis analysis in a fatal case of myelofibrosis complicated by recurrent candidemia that developed resistance during antifungal therapy.

Materials and results of pulsed-field gel electrophoresis

Case report

We treated a 45-year-old male with myelofibrosis complicated by candidiasis. Candida albicans was repeatedly isolated from blood cultures. The patient was treated with intravenous amphotericin B and intravenous fluconazole, as well as temporarily with flucytosine. Acid-fast bacilli were observed in smears of pharyngeal secretions and gastric juice. Intramuscular streptomycin was administered with oral rifampicin, isoniazid, ethambutol, and clarithromycin for about one month. Blood cultures were negative for C. albicans, and sputum cultures were negative for acid-fast bacilli. Echocardiography revealed thrombotic or tumor-like lesions in the right ventricle, suggesting endocarditis with verrucae, and C. albicans was repeatedly isolated. The patient died despite aggressive antifungal therapy with intravenous amphotericin B, intravenous fluconazole, and miconazole. Candida albicans was isolated ten times during the patient's hospital stay, and the total doses of antifungal agents administered were:
amphotericin B, 3,816 mg; fluconazole, 21,150 mg; miconazole, 10,800 mg; and flucytosine, 120 g.

The pathologic findings at autopsy were myelofibrosis, systemic hemosiderosis, and systemic candidiasis. The candidal lesions detected were macroscopically characterized by many verrucae filling the lumen of the root of the pulmonary artery and on the right ventricle wall. The verrucae proved to consist of clusters of fungal elements. Diffuse candidal microabscesses were seen in the myocardium of the left ventricle, both lower lobes of the lungs, and the pleura. Candidal lesions were also seen in the liver, kidneys, and spleen. There was little evidence of an inflammatory change in these lesions, however, the culture of the candidal lesions was not examined at autopsy. Details of this case have already been reported in Clinical Infectious Disease.6)

**Susceptibility testing** (Fig. 1)

*C. albicans* strains were repeatedly isolated from the patient's blood. The minimum inhibitory concentration (MIC) of antifungal agents for 10 strains of *C. albicans* isolated at various times during the clinical course was measured by standard susceptibility testing proposed by the National Committee for Clinical Laboratory Standards for yeast.7) The MIC of fluconazole for these strains increased from 0.125 μg/ml to over 64 μg/ml after 40 days of therapy with a total dose of more than 6 g. Moreover, during the same period, the MICs of miconazole and itraconazole increased from 0.031 μg/ml to 8 μg/ml, and from 0.008 μg/ml to over 8 μg/ml, respectively, even though these drugs were not used in treatment. The MIC of amphotericin B gradually increased from 0.063 μg/ml to 0.5 μg/ml. There was no change in MIC of flucytosine, which remained at 0.125 μg/ml after therapy.

**Analysis of chromosomal DNAs**

The chromosomal DNA of five fluconazole-sensitive *C. albicans* strains (nos. 1 through 5, MIC: 0.125 μg/ml) isolated during therapy in the first 4 weeks, five fluconazole-resistant *C. albicans* strains (nos. 6 through 10, MIC: >64 μg/ml) isolated during therapy in the second 7 weeks, and one *Saccharomyces cerevisiae*, strain was subjected to separation by pulsed-field gel electrophoresis using the methods of Merz et al.8) and

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Iwaguchi et al.9) (Fig. 2). Genetic approaches have proved valuable in defining strains of *S. cerevisiae* with specific genotypes and determining linkage groups and chromosome numbers.

The chromosomal DNA of all sensitive and resistant strains showed similar patterns, with no differences observed between the 1.0 and 2.0 Mb medium band, except for the strains having >2.0 Mb-bands. No bands were demonstrated in resistant strains Nos. 7, 8, or 9, suggesting a multiplicity of molecular weights. Similarly, resistant strains Nos. 6, 9, and 10 showed larger molecular bands.

**Discussion**

Risk factors for nosocomial candidiasis include prior treatment with multiple antibiotics, the presence of a central line, bladder catheterization, isolation of *Candida* species from sites other than the blood, azotemia or prior hemodialysis, transfer from other hospitals, diarrhea, and so forth.10)11) Particularly in patients with hematologic diseases, previous bacteremia, and prolonged neutropenia or fever, long-term antimicrobial therapy is jeopardized by serious risks of candidemia.12) In our patient, there were numerous risk factors prior to candidemia, including chemotherapy for myelofibrosis, multiple antimicrobial chemotherapy, indwelling intravenous catheters, prolonged neutropenia, and persistent fever unresponsive to broad-spectrum antibiotic therapy.

When administration of antibiotics permits overgrowth of *Candida* in the gastrointestinal tract, it may invade the intact or damaged intestinal mucosa3) and may cause *Candida* sepsis. In our case, sepsis resulted from an overgrowth of the yeasts due to use of multiple and broad-spectrum antimicrobial agents, as well as the use of an intravenous catheter.

Concomitant fluconazole and intravenous amphotericin B therapy was administered to this patient, and his fever transiently subsided. However, fungemia recurred because the heart lesion could not be resected and the endocardial lesions persisted. The repeatedly isolated strains of *C. albicans* were believed to be the same species.

Fluconazole has frequently been used for oropharyngeal candidiasis for long periods, and there are increasing reports of fluconazole-resistant *C. albicans* infections. However, fluconazole-resistant *Candida* infection is rare except in AIDS patients. Millon et al.3) reported that fluconazole-resistant *C. albicans* (MIC: >32 μg/ml) emerged after 90 days of treatment corresponding to a total dose of more than 10 g. The MIC of fluconazole for *C. albicans* isolated from our patient increased after a 40-day course corresponding to a total dose of more than 6 g. The MIC of amphotericin B for *C. albicans* was elevated after 70 days of treatment, corresponding to a total dose of more than 2 g.

The reasons for the development of resistance to fluconazole and the elevation of the MIC of amphotericin B for *C. albicans* are not clear, but the dose and/or the duration of the antifungal therapy may have been responsible.4) Thus, it would appear that azole resistance is an inevitable consequence of long-term therapy in permanently
immunosuppressed patients.

Among patients with acquired immunodeficiency syndrome (AIDS) who might benefit from continuous antifungal agents for esophageal candidiasis, those receiving chemotherapy for mycobacterial infections seem to be more prone to recurrence than others.\(^{15}\) Rifampicin has been reported to induce the metabolism of fluconazole.\(^{16}\) In our case, however, the serum fluconazole level determined by high-performance liquid chromatography was 8 μg/ml, when the patient was given fluconazole at 200 mg/day and rifampicin at 450 mg/day, in contrast to 16 to 17.2 μg/ml when he was given fluconazole monotherapy at 400 mg/day. Thus, no induction of fluconazole metabolism by rifampicin was observed. Further study is necessary to determine drug interactions during continuous, concomitant administration of antifungal agents and antimycobacterial agents.

It is generally believed that resistance can develop in clinical C. albicans isolates as a result of changes in permeability of the fungal cell wall, overexpression of different multidrug efflux transporter genes, or affinity alteration to azole antifungal agents through specific mutations of their target.

Acquired cross-resistance to azole antifungal agents is caused mainly by overexpression of a distinct multidrug efflux transporter gene, while intrinsic low levels of resistance to fluconazole seem to be due to the constitutive expression of another transporter gene.\(^{17-20}\)

We used the pulsed-field gel electrophoresis method to elucidate the difference of karyotype between fluconazole-sensitive and fluconazole-resistant strains of C. albicans. The results showed the same karyotype among all strains isolated during the clinical course; there were no major differences in DNA profile. This suggests that the C. albicans strains isolated from our patient which were causing the continued fungemia and the strains that were sensitive to fluconazole therapy were closely related. These data showed that repeatedly isolated C. albicans were the same strain and acquired a resistance to antifungal agents during the therapy. Moreover, the presence of the new bands in strains Nos. 6, 9, and 10, and the lack of bands in strains Nos. 7, 8, and 9 hinted at the effects of slight changes in DNA. These changes may suggest a mechanism of acquisition of resistance of C. albicans to these antifungal agents. However, these results were obtained by analyzing only one case, and further studies on resistance mechanisms will be necessary for confirmation.

References