Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*

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Running Title: Clinical azole exposure and azole MIC for *Aspergillus*

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ABSTRACT

This is the first report of a detailed relationship between triazole treatment history and triazole MICs for 154 *Aspergillus fumigatus* clinical isolates. The duration of itraconazole dosage increased as the itraconazole MIC increased, and a positive correlation was observed ($r = 0.5700$, $p < 0.0001$). The number of itraconazole-naïve isolates dramatically decreased as the itraconazole MIC increased, particularly for MICs exceeding 2 μg/ml (0.5 μg/ml vs. 2 μg/ml, $p = 0.03$). We also examined the relationship between cumulative itraconazole usage and the MICs of other azoles. A positive correlation existed between itraconazole dosage period and posaconazole MIC ($r = 0.5237$, $p < 0.0001$). The number of itraconazole-naïve isolates also decreased as the posaconazole MIC increased, particularly for MICs exceeding 0.5 μg/ml (0.25 μg/ml vs. 0.5 μg/ml, $p = 0.004$). Conversely, the correlation coefficient obtained from the scattergram of itraconazole usage and voriconazole MICs was small ($r = -0.2627$, $p = 0.001$). Susceptibility to three triazole agents did not change as the duration of voriconazole exposure changed. In addition, we carried out detailed analysis, including microsatellite genotyping, for isolates obtained from patients infected with azole-resistant *A. fumigatus*. We confirmed the presence of acquired resistance to itraconazole and posaconazole due to a G54 substitution in the *cyp51A* gene for a

1 patient with chronic pulmonary aspergillosis after oral itraconazole therapy. We should
2 consider the possible appearance of azole-resistant *A. fumigatus* if itraconazole is used
3 for extended periods.
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INTRODUCTION

Aspergillosis has become an increasingly important fungal infection because the number of immunocompromised patients has increased (21, 29). However, antifungal drugs for treating different types of aspergillosis such as invasive pulmonary aspergillosis or chronic pulmonary aspergillosis have insufficient efficacy (18-20, 32). Among the few types of drugs with anti-*Aspergillus* activity, triazoles hold a prominent position because they are the only licensed class of oral drugs for treating aspergillosis (32).

Recently, the appearance of azole-resistant *Aspergillus fumigatus* has come under scrutiny in several countries (1, 2, 7, 14, 17, 23-27, 30). Reports from some countries have raised concerns over the increased prevalence of azole-resistant *A. fumigatus* (7, 17, 27). Therefore, it is important to elucidate the mechanism of resistance to prevent the spread of azole-resistant *A. fumigatus* and subsequent outbreaks. The possible origins of these azole-resistant isolates include the environment and the patient's own body (31). Some cases of acquired resistance in *A. fumigatus* have been reported in patients with aspergilloma during treatment with azoles (3, 6, 8, 9, 11, 22). Environments such as farms are especially suspected of promoting the production of azole-resistant isolates harboring the TR/L98H mutation in the *cyp51A* gene, which encodes cytochrome P450

14-α sterol demethylase, the primary target for azole compounds (23, 31).

Despite the presence of case reports on the development of azole resistance during azole therapy, little information is available on the amount of azole needed for the development of azole resistance (8, 17, 22). Howard et al. reported that the first azole-resistant isolate was identified after using azole for 1–30 months (17). Recent study by Camps et al. raised warning of a rapid induction of resistance for which the median time between isolation of the last cultured wild-type isolate until the first azole-resistant isolate was 4 month (8). Such data are important because long-term, perhaps lifelong, antifungal treatment is required for some chronic pulmonary aspergillosis cases (32).

Recently, we reported the antifungal MIC distribution of 196 *A. fumigatus* clinical isolates with *cyp51A* gene mutation in Nagasaki, Japan (28). Of these, we analyzed 154 isolates from 64 patients retrospectively in this study, and we evaluated the cumulative amount of azoles administered to patients at the time of isolation of each *A. fumigatus* clinical isolate. Moreover, we investigated the backgrounds of patients from whom azole-resistant *A. fumigatus* was isolated and conducted microsatellite genotyping of the isolates to analyze their genetic relationships. This is the first report to analyze the correlation between azole usage and azole susceptibility of *A. fumigatus* clinical

isolates.

MATERIALS AND METHODS

***A. fumigatus* isolates** The isolates were collected in the Pneumology Department of Nagasaki University Hospital, Nagasaki, Japan between February 1994 and April 2010. We identified all isolates as *A. fumigatus* according to the macroscopic colony morphological and micromorphological characteristics, and the ability to grow at 48°C (4). Azole-resistant isolates were subjected to additional molecular identification by amplification of ribosomal internal transcribed spacers and ribosomal large-subunit D1-D2 sequencing as described previously (16).

Patients Clinical information was extracted from the clinical records on the type of aspergillosis and history of azole antifungal use. The periods of triazole administration were cumulatively determined until the time of *A. fumigatus* isolation; therefore, the periods were different for each isolate and even for isolates obtained from the same patient. In patients infected with azole-resistant *A. fumigatus*, we examined the underlying diseases and characteristics of therapeutic failure. Patient 1 (48-year-old man) had chronic cavitary pulmonary aspergillosis (CCPA) (Table 2). Both his lungs were damaged by multiple partial lobectomies because of repeated refractory

pneumothorax, and multiple cavities and bullas with pleural thickness were observed in both the lungs. *A. fumigatus* was frequently cultured from his sputum despite oral itraconazole treatment (200–400 mg/day). After the isolation of itraconazole-resistant *A. fumigatus*, the patient was treated with oral voriconazole. Since then, his symptoms such as productive cough or hemosputum have improved, and no fungus has been subsequently isolated from his sputum. Patient 2 (70-year-old woman) was clinically diagnosed as having aspergilloma in the upper lobes of both the lungs (Table 2). She had a history of pulmonary tuberculosis and had several cavities in both the lungs. Patients 3 (80-year-old woman) and Patient 5 (63-year-old man) were diagnosed with simple aspergilloma. Patient 4 (56-year-old woman) were diagnosed with CCPA (Table 2).

Antifungal susceptibility testing and *cyp51A* sequencing We previously reported the results for antifungal susceptibility and *cyp51A* sequencing (28). The breakpoints used for resistance were ≥4 μg/ml for itraconazole and voriconazole and≥1 μg/ml for posaconazole (30).

Genotyping Sixteen isolates (including both azole-susceptible and azole-resistant isolates) were obtained from 5 patients infected with azole-resistant *A. fumigatus*. DNA was extracted from these isolates by using the MasterPure yeast DNA purification kit

(Epicentre Biotechnologies, Madison, WI), and 9 short tandem repeat region (2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C) were amplified by PCR as described previously (12). The repeat numbers were determined by sequencing analysis, and we compared the patterns of repeat numbers. DNA sequences were determined using a BigDye Terminator version 1.1 cycle sequencing kit (ABI, USA) and an ABI 3100xl DNA analyzer.

Statistics Statistical analyses of azole usage and azole susceptibility were performed using Pearson's correlation and Fisher's exact tests with Prism version 5.0 (GraphPad, USA). Differences were considered significant when $p < 0.05$.

RESULTS

Correlation between azole usage (duration and amount) and azole susceptibility A total of 154 *A. fumigatus* clinical isolates obtained from 64 patients were analyzed. Most of these specimens were isolated from the lungs (Table 1). Chronic pulmonary aspergillosis (included simple aspergilloma) accounted for 61% of the clinical diagnoses (Table 1).

The scatter plot of the itraconazole dosage period and itraconazole MICs is shown in Figure 1A. Patients infected by *A. fumigatus* with itraconazole MICs < 2 μg/ml had been treated with itraconazole for <1 year. All isolates with itraconazole MICs ≥ 4

μg/ml (MF-452, MF-460, MF-468, MF-469, MF-329, and MF-357) had been exposed to itraconazole for >115 days (Table 2). The itraconazole dosage duration increased as the itraconazole MIC increased, and the dosage duration was positively correlated with the itraconazole MIC (r = 0.5700, p < 0.0001) (Figure 1A). The number of itraconazole-naïve isolates dramatically decreased as the MIC increased, particularly for MICs exceeding 2 μg/ml (0.5 μg/ml vs. 2 μg/ml, p = 0.03) (Figure 1B). These results indicated that long-term itraconazole treatment could induce azole-resistant *A. fumigatus*.

A positive correlation was also observed between the itraconazole dosage period and the posaconazole MIC (r = 0.5237, p < 0.0001) (Figure 2A). The number of itraconazole-naïve isolates decreased as the posaconazole MIC increased, particularly for posaconazole MICs exceeding 0.5 μg/ml (0.25 μg/ml vs. 0.5 μg/ml, p = 0.004) (Figure 2C). The correlation coefficient obtained from the scattergram of itraconazole usage and voriconazole MICs was small (r = −0.2627, p = 0.001) (Figure 2B). The voriconazole MIC did not increase with increasing itraconazole usage. In addition, the numbers of itraconazole-naïve isolates was not correlated with the voriconazole MIC (Figure 2D). These results suggested the possibility of inducing resistance to posaconazole but not to voriconazole by long-term itraconazole therapy.

A. fumigatus was isolated after voriconazole treatment from only a few patients; therefore, an analysis of the relationship between voriconazole usage histories before *A. fumigatus* isolation and azole susceptibilities was limited. Only 10 isolates were exposed to voriconazole therapy before isolation, and the average duration of the therapy was 8.3 ± 6.3 days. Voriconazole exposure did not alter the susceptibility of the 3 triazole agents.

In this study, we counted the duration of azole exposure as the cumulative time of treatment. *A. fumigatus* was not always clinically isolated from patients during therapy; it was also isolated after the cessation of azole therapy. Because the selection pressure on azole-resistant *A. fumigatus* might be the highest during the treatment, azole resistance might dissipate over time after therapy. Hence, we examined the relationship between the itraconazole MIC and the time from the end of itraconazole therapy to isolation. Of the 154 isolates, 42 had been exposed to itraconazole therapy before isolation. The time from the end of itraconazole treatment to isolation had no relationship with itraconazole susceptibility ($r = -0.1302$, $p = 0.4110$) (Figure 3). Azole-resistant *A. fumigatus* was isolated even after azole treatment had been discontinued.

Clinical analysis of patients infected with azole-resistant *A. fumigatus* Five

patients were infected with azole-resistant *A. fumigatus*, and 16 isolates were obtained from these patients (including susceptible isolates) (Table 2). To analyze the genetic relationships among these 16 isolates, a panel of nine short tandem repeats for exact and high-resolution fingerprinting of *A. fumigatus* isolates was performed in this study. The 16 isolates obtained from the 5 patients were divided into 6 genotypes via microsatellite typing (Table 3).

Nine isolates were cultured from Patient 1 (Table 2). *A. fumigatus* isolated in earlier periods was azole-susceptible, and it harbored the I266N mutation in the *cyp51A* gene; however, later isolates showed itraconazole or posaconazole resistance and new mutations such as G54E. Despite the discontinuation of itraconazole treatment, azole-resistant isolates were cultured from his sputum 140 days after the end of the treatment (Table 2). All isolates were confirmed to be genetically homogeneous (Table 3).

In Patient 2, three *A. fumigatus* isolates were cultured during days 115–132 of the itraconazole dosage period. The isolates were homogeneous; however, the itraconazole or posaconazole MICs and *cyp51A* mutations in the three isolates were significantly different (Tables 2–3). *A. fumigatus* isolates from Patient 4 were heterogeneous.

DISCUSSION

In this study, we showed a correlation between the duration of clinical itraconazole exposure and the MICs of triazoles for *A. fumigatus*. It has already been reported that itraconazole exposure can induce the formation of azole-resistant *A. fumigatus* carrying a G54 mutation in the *cyp51A* gene in vitro (13). As expected, increased used of itraconazole was associated with decreased itraconazole susceptibility among the *A. fumigatus* clinical isolates. The posaconazole susceptibility of the isolates was also decreased, presumably because of the appearance of G54 substitution in the *cyp51A* gene, indicating that clinicians should be careful when selecting posaconazole as an antifungal agent for the treatment of patients who have previously received long-term itraconazole therapy. If long-term itraconazole therapy induces voriconazole resistance in *A. fumigatus*, then this will have a significant impact on the treatment of aspergillosis. Our study indicated that itraconazole treatment did not induce voriconazole cross-resistance. These results were consistent with previous reports (15, 25). The reason for the lack of cross-resistance between itraconazole and voriconazole in this study was that the G54 mutation in azole-resistant isolates resulted in a resistance to itraconazole and posaconazole but not to voriconazole.

The most important limitation of this study was that no data could be obtained regarding the serum concentration of itraconazole during its usage. Itraconazole has a relatively low bioavailability after oral administration, especially when given in capsule form (33). Of the 42 isolates exposed to itraconazole before isolation, 39 had been exposed to itraconazole capsules, and the remaining 3 isolates had been exposed to the oral solution, which has a greater bioavailability than the capsule form (5). Most patients who were administered the capsule form of itraconazole were prescribed a dose of 200 mg/day, which is the approved dose in Japan. Despite the lack of a report examining the presence of a mutation selection window for itraconazole by *A. fumigatus*, both the low bioavailability and blood concentration of itraconazole in capsule form might be risk factors for azole resistance. The solution form may overcome these disadvantages; however, Patient 4 who was infected with posaconazole-resistant *A. fumigatus* carrying the G54W *cyp51A* mutation, had been administered the itraconazole oral solution at a dose of 200 mg/day for 210 days.

Itraconazole oral therapy is often administered long-term for the treatment of chronic pulmonary aspergillosis (32). The judgment of treatment failure is still difficult; therefore, we need more information to decide whether the itraconazole treatment should be continued. Despite the importance of the duration of itraconazole treatment

with respect to the induction of azole resistance, few studies have investigated the relationship between azole resistance and azole exposure. Howard et al. reported that the duration of azole exposure before the identification of the first resistant isolate was 1–30 months, and the most commonly administered azole was itraconazole (17). Mortensen et al. also reported that patients with azole-resistant *A. fumigatus* isolates had received mold-active azoles for 11.5–69.5 months before the detection of resistant isolates (22). In our study, patients with azole-resistant *A. fumigatus* had been administered itraconazole for 3.8–24.3 months. These data are similar to those described above. Moreover, patients infected by *A. fumigatus* with itraconazole MICs < 2 μg/ml had been administered itraconazole for <1 year. Clinicians should be careful of the potential appearance of itraconazole-resistant isolates during long-term sequential itraconazole therapy for several months to more than 1 year.

Recently, Camps et al. reported that median time between the last cultured wild-type isolate and the first azole-resistant isolate was 4 month (range, 3 weeks to 23 months) (8). In our study, time between the last isolation of azole sensitive strain and first appearance of azole-resistant strain was about 10 and 7 months in patient 1 and 4, respectively (Table 2). These periods were longer than median time reported by Camps et al. while fell within reported range (3 weeks to 23 months).

We confirmed that long-term itraconazole therapy induced azole resistance in *A. fumigatus*. Even if azole-resistant mutants were dominant during treatment, their dominance could dissipate after cessation of the therapy because of the differences in the growth rates of the resistant and susceptible specimens (3). However, resistant isolates were still cultured 140 days after the cessation of azole therapy in Patient 1. In Patients 3 and 5, the time from the end of treatment to isolation was 1223 and 435 days, respectively, which might indicate the possibility of the presence of resistant isolates for years after the end of azole therapy or the possibility of new infection. There were no differences in the growth rate of azole-resistant and azole-susceptible *A. fumigatus* isolates in vitro (data not shown). When patients receive long-term itraconazole therapy, clinicians should aggressively culture *A. fumigatus* from the patients and perform susceptibility tests even long after the cessation of itraconazole therapy.

We isolated azole-resistant *A. fumigatus* from clinical samples, such as sputum, but we did not isolate *A. fumigatus* from the environment or detect a TR/L98H mutant (28). It is interesting to note that the most common mechanism of resistance detected in this study was G54 substitution, because the selection pressure of itraconazole induces G54 mutation (13). Moreover, most resistant isolates detected in the environments around the world carry the TR/L98H substitution and no other mutation such as G54

substitution (10, 23). These facts suggest that different azoles select different mutations. Itraconazole might selectively induce mutations such as G54 substitution, whereas some azoles used in agriculture may tend to select the TR/L98H mutation. The mechanisms of these differences remain to be completely elucidated. Further investigation is needed to clarify these mechanisms, and this knowledge may enable us to prevent the induction of the TR/L98H mutation in the environment.

In conclusion, this is the first report to show a detailed relationship between azole usage and azole MICs for *A. fumigatus*. Furthermore, we confirmed the presence of acquired resistance to itraconazole and posaconazole in a patient with chronic pulmonary aspergillosis after consecutive oral itraconazole therapy in Japan. The possibility of azole-resistant *A. fumigatus* should be considered during long-term itraconazole therapy in patients with chronic pulmonary aspergillosis.

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FIGURE LEGENDS

Figure 1. Relationship between itraconazole MICs and the history of itraconazole usage for 154 *A. fumigatus* clinical isolates. (A) The itraconazole dosage duration increased as the itraconazole MIC increased, and a positive correlation was observed between the itraconazole dosage duration and the itraconazole MIC (r = 0.5700, p < 0.0001). (B) The number of itraconazole-naïve isolates dramatically decreased as the itraconazole MIC increased, particularly for itraconazole MICs exceeding 2 μg/ml (0.5 μg/ml vs. 2 μg/ml, p = 0.03). *p < 0.05 (Fisher's exact test).

Figure 2. Relationship between the MICs of other triazoles and the history of itraconazole usage for the 154 *A. fumigatus* clinical isolates. (A) A positive correlation was observed between the itraconazole dosage period and the posaconazole MIC (r = 0.5237, p < 0.0001) (B) The number of itraconazole-naïve isolates decreased as the posaconazole MIC increased, particularly for posaconazole MICs exceeding 0.5 μg/ml (0.25 μg/ml vs. 0.5 μg/ml, p = 0.004). (C) The correlation coefficient obtained from the scattergram of itraconazole usage and voriconazole MICs was small (r = −0.2627, p =0.001). (D) No significant difference was observed in the percentage of itraconazole-naïve isolates and the individual MICs of voriconazole. *p < 0.05 (Fisher's

exact test).

Figure 3. We examined the relationship between itraconazole MICs and the time from the end of itraconazole therapy to *A. fumigatus* isolation. Of the 154 isolates, 42 had been exposed to itraconazole before isolation. These isolates were analyzed for the relationship; however, the relationship could not be confirmed by the scatter plot ($r = -0.1302$, $p = 0.4110$).

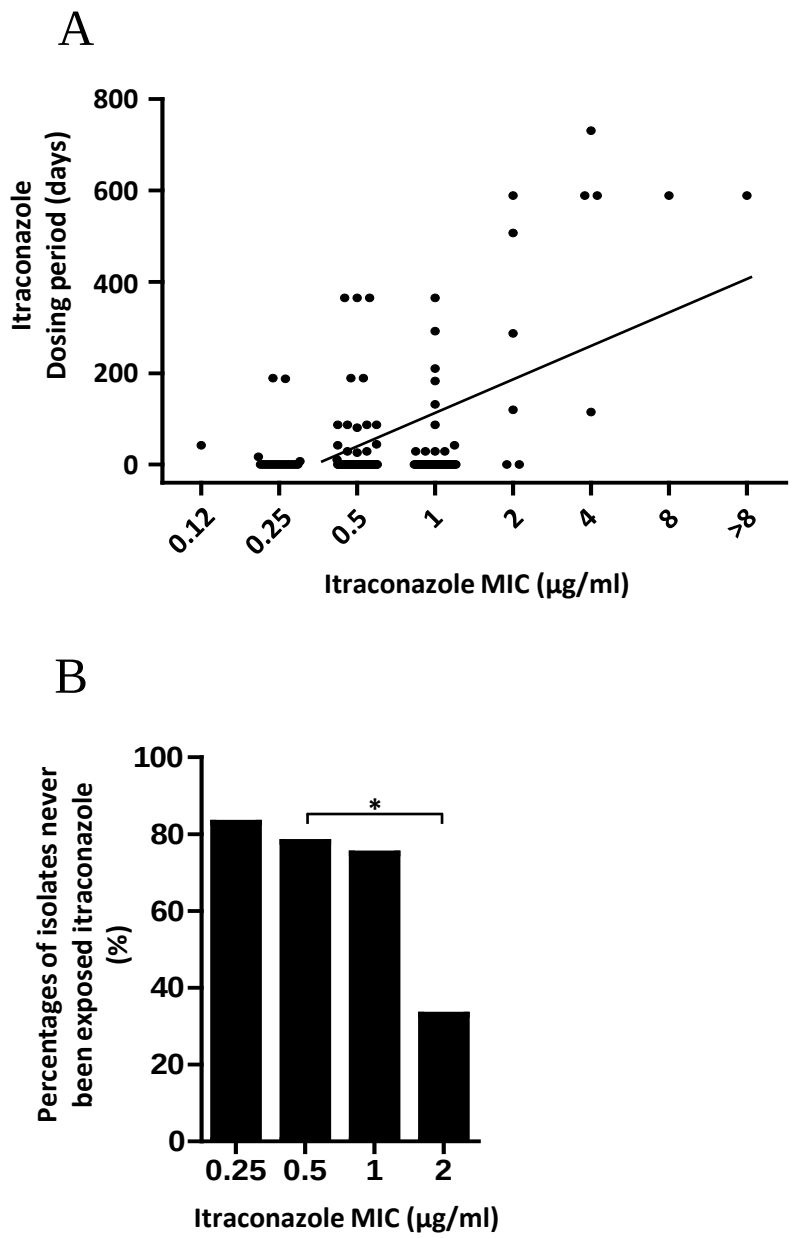

Figure 1. Tashiro et al.

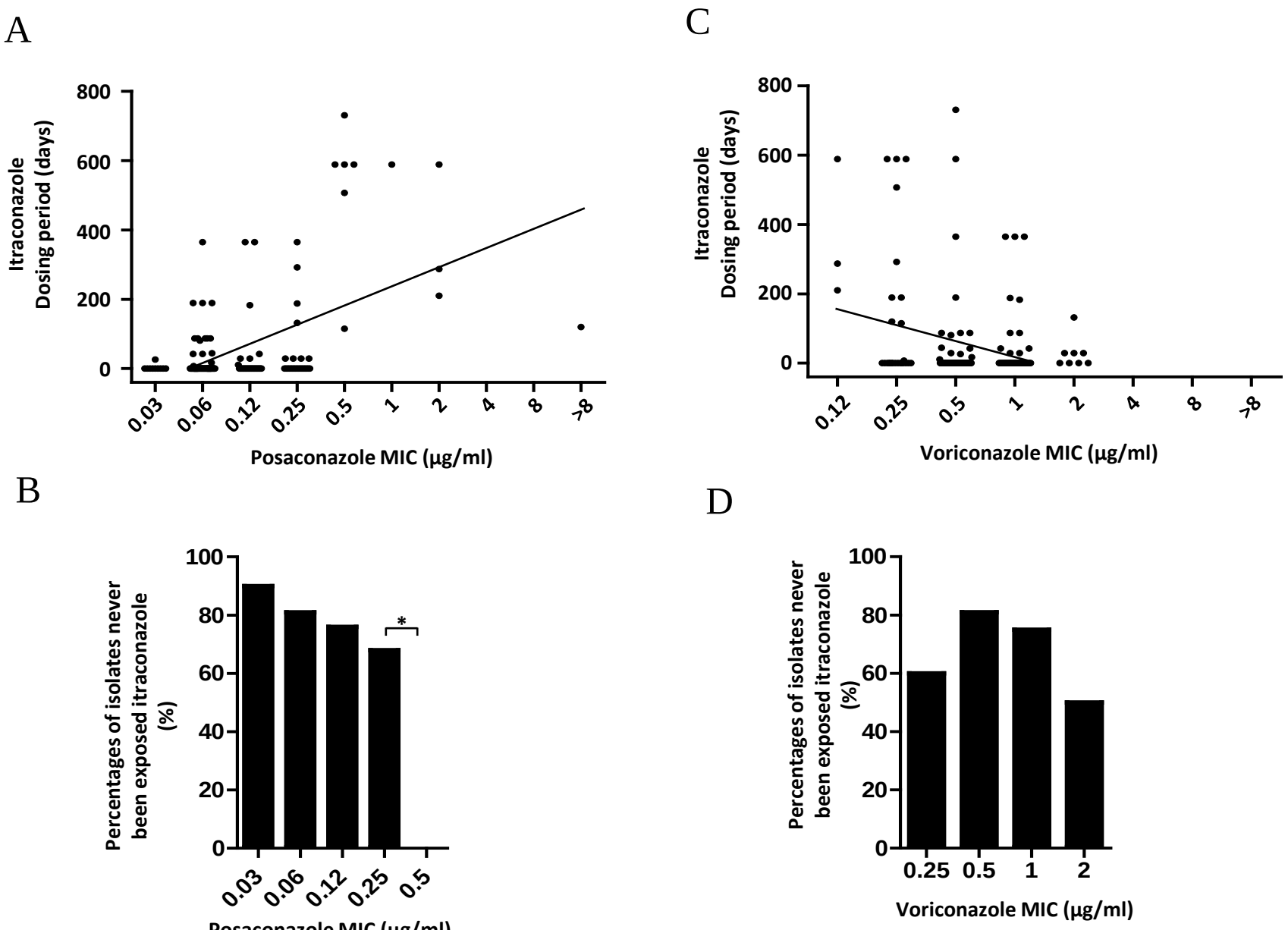

Figure 2. Tashiro et al.

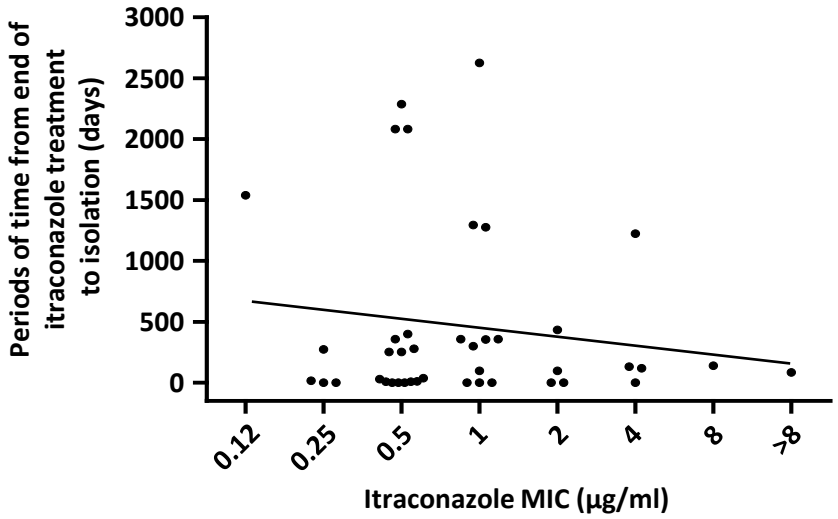

Figure 3. Tashiro et al.

TABLE 1. Characteristics of patients and isolates

Isolates	154
Patients	64
Sample origin, n (%)	
Sputum	96/154 (62)
Bronchoalveolar lavage fluid	36/154 (23)
Lung tissue	9/154 (5.8)
Others [a]	2/154 (1.3)
Unknown	11/154 (7.1)
Clinical diagnosis [b], n (%)	
Invasive pulmonary aspergillosis [c]	9/64 (14)
Chronic pulmonary aspergillosis except simple aspergilloma	27/64 (42)
Simple aspergilloma	12/64 (19)
Allergic bronchopulmonary aspergillosis	4/64 (6.3)
Colonization	12/64 (19)

[a] Others include lung abscess and bone marrow.
[b] Diagnosis of other 23 patients were unknown.
[c] All were diagnosed as probable.

Table1. Tashiro et al.

TABLE 2. Characteristics of the 16 isolates obtained from patients infected with azole-resistant *A. fumigatus* [a]

Patient no.	Isolate no.	Date of isolation (day-mo-yr)	ITC exposure [b]		Time from end of ITC therapy (days)	MIC (μg/ml) [c]			Cyp51A substitution [d]
			Periods (days)	Amounts (mg)		ITC	POS	VRC	
1	MF-368	16-08-2000	189	37,800	252	0.5	0.06	0.5	No substitution
	MF-367	16-08-2000	189	37,800	252	0.5	0.06	0.25	No substitution
	MF-370	07-09-2000	189	37,800	274	0.25	0.06	0.25	No substitution
	MF-439	19-10-2001	507	144,850	0	2	0.5	0.25	G54E
	MF-452	03-04-2002	589	161,650	84	>8	0.5	0.5	No substitution
	MF-454	17-04-2002	589	161,650	98	2	0.5	0.125	G54E
	MF-460	08-05-2002	589	161,650	119	4	2	0.25	G54E
	MF-468	22-05-2002	589	161,650	133	4	0.5	0.25	G54E
	MF-469	29-05-2002	589	161,650	140	8	1	0.25	G54E
2	MF-329	24-08-1998	115	23,000	0	4	0.5	0.25	No substitution
	MF-331	29-08-1998	120	24,000	0	2	>8	0.25	G54W
	MF-336	10-09-1998	132	26,400	0	1	0.25	2	No substitution
3	MF-357	09-02-2000	731	146,200	1223	4	0.5	0.5	No substitution
4	MF-933	11-03-2008	0	0	-	0.5	0.25	0.25	No substitution
	MF-1011	09-10-2008	210	42,000	0	1	2	0.125	G54W
5	MF-327	16-07-1998	287	43,050	435	2	2	0.125	G54R

[a] Azole-resistant *A. fumigatus* had itraconazole MIC ≥4μg/ml or posaconazole MIC ≥1μg/ml. Voriconazole resistant isolates (Voriconazole MIC ≥4μg/ml) were not found.

[b] Accumulated periods and amounts before isolation.

[c] ITC, itraconazole; POS, posaconazole; VRC, voriconazole.

[d] Only substitution associated with azole resistance.

Table 2. Tashiro et al.

TABLE 3. Genotypes of the 16 *A. fumigatus* isolates by STR*Af*

Patient no.	Isolate no.	STR*Af* [a]								
		2A	2B	2C	3A	3B	3C	4A	4B	4C
1	MF-368	23	15	10	25	11	32	8	10	7
	MF-367	23	15	10	25	11	32	8	10	7
	MF-370	23	15	10	25	11	32	8	10	7
	MF-439	23	15	10	25	11	32	8	10	7
	MF-452	23	15	10	25	11	32	8	10	7
	MF-454	23	15	10	25	11	32	8	10	7
	MF-460	23	15	10	25	11	32	8	10	7
	MF-468	23	15	10	25	11	32	8	10	7
	MF-469	23	15	10	25	11	32	8	10	7
2	MF-329	19	21	14	18	10	16	7	13	5
	MF-331	19	21	14	18	10	16	7	13	5
	MF-336	19	21	14	18	10	16	7	13	5
3	MF-357	18	19	23	34	13	20	18	9	8
4	MF-933	20	12	20	24	22	36	13	9	5
	MF-1011	11	21	11	28	12	31	18	9	10
5	MF-327	21	21	10	23	11	27	8	9	8

[a] Number of tandem repeats at the given microsatellite number.

Table 3. Tashiro et al.