First clinical isolation report of azole-resistant *Aspergillus fumigatus* with TR_{34}/L98H-type mutation in Japan

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Abstract

Recently, azole-resistant *Aspergillus fumigatus* containing a 34-bp or 46-bp tandem repeat in the promoter region of *cyp51A* combined with amino acid substitution(s) has appeared in the environment worldwide, including several Asian countries. In this study, we isolated the 34-bp tandem repeat-containing azole-resistant *A. fumigatus* strain OKH50 from a patient in Japan in May 2016. The patient had not been treated with medical azoles before the strain isolation, suggesting that the resistant property was acquired before infection. In addition, the patient had not traveled overseas. Our analysis of short tandem repeats of the strain indicates that the strain is strongly related to the 34-bp tandem repeat-containing isolates from European countries and Asia-Oceania countries but not to susceptible isolates from Japan, suggesting that the strain was introduced from overseas and might spread in Japan.

Keywords: azole, *Aspergillus fumigatus*, *cyp51A*, TR_{34}/L98H

Aspergillus fumigatus is a commonly found fungus in the environment and is known to be the leading causative agent of aspergillosis. Although azole antifungals are widely used to treat aspergillosis, an azole-resistant strain of *A. fumigatus* has recently appeared. Two mechanisms for the acquisition of azole resistance are the selection route in a patient and that in the environment [1].

Most of the azole-resistant isolates found in the environment harbor the *cyp51A* mutation consisting of a tandem repeat in the *cyp51A* promoter region and amino acid substitution(s) named as TR_{34}/L98H [2] or TR_{46}/Y121F/T289A [3]. Hagiwara and colleagues recently reported the first Japanese case of isolation of TR_{46}/Y121F/T289A [4]. However, the TR_{34}/L98H mutant has not been reported in Japan. In this manuscript, we report the first case of isolation of the TR_{34}/L98H mutant of *A. fumigatus* in Japan.

The *Aspergillus fumigatus* OKH50 strain was isolated from the sputum of a 77-year old male with a tumor at a hospital in Obihiro City in May 2016. He had been a government worker, with a hobby for gardening; he lived in a housing complex surrounded by agricultural land in Obihiro City. The patient had a past history of hepatitis C followed by liver cirrhosis. The illness included a hepatocellular carcinoma, bronchial asthma, and type 2 diabetes, and the patient had not been diagnosed with aspergillosis when OKH50 was isolated. During the treatment of the hepatocellular tumor, wet cough and low-grade fever

appeared. Aspergillosis was not diagnosed initially because of the absence of typical findings of pulmonary aspergillosis on chest X-ray photography. After isolation from a sputum sample, the strain was visually identified as *A. fumigatus*, and the growth and conidia formation were not distinguished from other susceptible strains of *A. fumigatus* (data not shown); consequently, the patient was diagnosed with aspergillosis. In addition, the patient's serum showed high titer against *Aspergillus* antigen in an immunodiffusion test (data not shown). Genomic DNA was extracted from the clinical isolate and identified as *A. fumigatus* by determining the internal transcribed spacer and D1/D2 regions, and partial nucleotide sequences of β-tubulin, *rodA*, and calmodulin genes. Simultaneously, the minimal inhibitory concentrations (MICs) against *A. fumigatus* OKH50 were determined by the microdilution method based on CLSI M38-A2. The strain OKH50 revealed a resistance to voriconazole (MIC, 4 or 8 μg/mL) and itraconazole (MIC, >8 μg/mL).

To identify mutations in *cyp51A* and the upstream regions of *A. fumigatus* OKH50, we determined the following nucleotide sequences: 34-bp tandem repeats in the *cyp51A* promoter region and L98H mutation in Cyp51A. The repeats in the promoter region of OKH50 were located at the identical site to those in previously reported sequences possessing 34-bp tandem repeats (Figure 1).

Next, to examine the dendrogram analysis based on short tandem repeat (STR)

typing, we determined STRs and analyzed them as previously described [4,5]. As shown in Figure 2, the OKH50 strain was clustered with TR34/L98H strains isolated in the Netherlands, Australia, Denmark, India, Southern Taiwan, and Kuwait. On the other hand, the OKH50 strain was not clustered around susceptible *A. fumigatus* strains isolated in Japan. T18_R strain and Iranian strains including Hamid 02 in the cluster were isolated from soil samples in Denmark and Iran, respectively [6, 7]. These data suggest that soil containing azole-resistant strains traveled with human or material, or were dispersed by air current. The patient had not traveled overseas, suggesting that the OKH50 or parental strain was introduced from overseas and might spread in Japan. In our previous study, neither the TR34/L98H- nor the TR46/Y121F/T289A-types were isolated from soil samples in Tokachi area [8]; therefore, we need to maintain continuous surveillance of *A. fumigatus* isolates in Japan, including Hokkaido.

Acknowledgments

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Conflict of Interest

None

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144 Figure Legends

145 **Fig. 1** Nucleotide sequences of *A. fumigatus cyp51A* promoter region of Af293 (wild type),

146 OKH50, and three other TR_{34}/L98H strains

147 The first boxed site indicates the original 34-bp region. The second boxed site indicates the

148 34-bp repeat region. Grey-shaded sites indicate the start codon.

149 **Fig. 2** Dendrogram of 34 *A. fumigatus* strains based on STR typing

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Figure 1

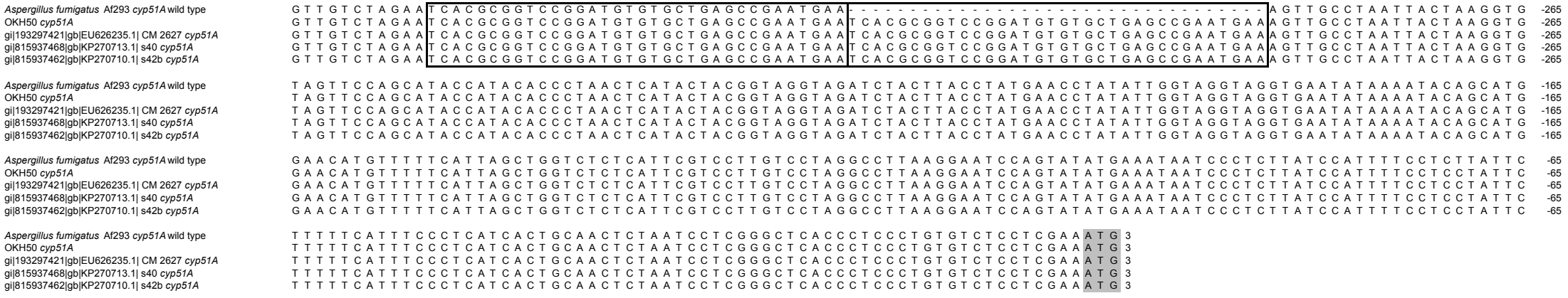
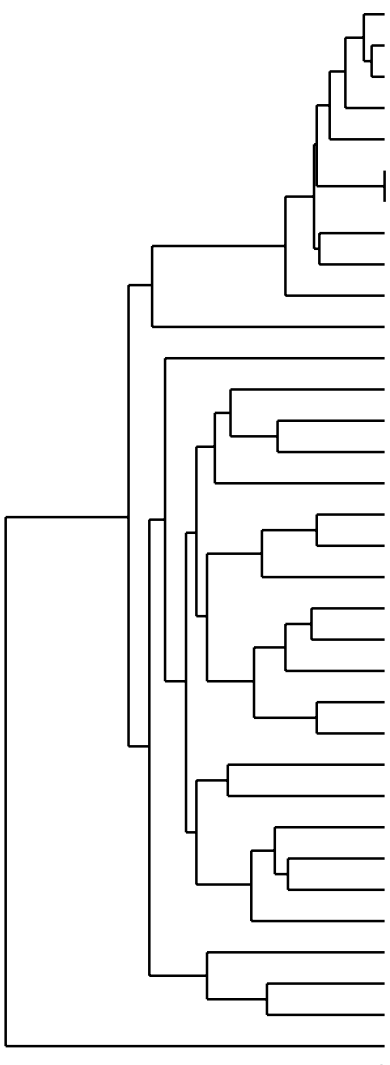

Aspergillus fumigatus Af293 cyp51A wild type
OKH50 cyp51A
gi|193297421|gb|EU626235.1| CM 2627 cyp51A
gi|815937468|gb|KP270713.1| s40 cyp51A
gi|815937462|gb|KP270710.1| s42b cyp51A
GTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAA----------AGTTGCCTAATTACTAAGGTG -265
TAGTTCCAGCATACCATACACCCTAACTCATACTACGGTAGGTAGATCTACTTACCTATGAACCTATATTGGTAGGTAGGTGAATATAAAATACAGCATG -165
GAACATGTTTTTCATTAGCTGGTCTCTCATTCGTCCTTGTCCTAGGCCTTAAGGAATCCAGTATATGAAATAATCCCTCTTATCCATTTTCCTCCTTATTC -65
TTTTTCATTTCCCTCATCACTGCAACTCTAATCCTCGGGCTCACCCTCCCTGTGTCTCCTCGAAATG 3

Figure 2



2A	2B	2C	3A	3B	3C	4A	4B	4C	Strain ID	Phenotype (mutation)	Country	Reference
14	21	8	28	9	6	8	10	18	OKH50	Resistant (TR_{34}/L98H)	Japan	This study
14	23	8	30	9	6	8	10	20	2005-456307L	Resistant (TR_{34}/L98H)	The Netherlands	9
14	21	8	31	9	6	8	10	20	04-202165	Resistant (TR_{34}/L98H)	Australia	10
10	20	8	32	9	7	8	9	19	T18_R	Resistant (TR_{34}/L98H)	Denmark	6
14	20	8	32	9	6	8	10	20	Hamid 02	Resistant (TR_{34}/L98H)	Iran	7
14	20	8	40	9	11	8	10	20	2005-456473	Resistant (TR_{34}/L98H)	The Netherlands	9
14	20	8	40	9	11	8	10	20	R5-07-4_R	Resistant (TR_{34}/L98H/S297T/F495I)	Denmark	6
14	20	9	31	9	10	8	10	28	1042/09	Resistant (TR_{34}/L98H)	India	9
20	21	10	31	11	10	8	10	20	A31	Resistant (TR_{34}/L98H)	Taiwan	12
20	10	8	35	9	11	8	10	24	Kw1431	Resistant (TR_{34}/L98H)	Kuwait	10
13	10	8	72	9	9	8	9	9	094411/7/50	Resistant (TR_{34}/L98H)	The Netherlands	10
18	23	15	38	11	48	10	9	8	18512R	Resistant (TR_{34}/L98H)	France	7
18	20	15	42	24	6	9	10	10	20643.017	Resistant (TR_{34}/L98H/S297T/F495I)	China	11
25	20	19	31	9	10	10	14	5	12-90032258	Resistant (TR_{34}/L98H)	Australia	10
25	16	13	33	19	10	10	9	8	IFM 59056	Susceptible	Japan	4
12	16	15	23	18	12	12	10	0	B60	Susceptible	Taiwan	12
26	12	11	22	10	26	12	10	10	20684.022	Resistant (TR_{34}/L98H/S297T/F495I)	China	11
23	12	11	23	23	26	10	10	10	20677.079	Resistant (TR_{34}/L98H/S297T/F495I)	China	11
25	20	20	23	22	33	10	11	10	94/P/10	Susceptible	India	7
18	24	20	22	13	20	10	10	8	A24	Susceptible	Taiwan	12
19	25	29	22	10	15	10	9	8	OKH31	Susceptible	Japan	8
17	22	20	23	23	11	10	9	8	IFM 60514	Susceptible	Japan	13
17	25	22	27	13	28	10	9	5	IFM 59365	Susceptible	Japan	13
17	22	22	31	11	26	18	9	5	IFM 61407	Susceptible	Japan	13
19	23	14	30	28	26	12	9	15	B51	Resistant (TR_{34}/L98H/S297T/F495I)	Taiwan	12
19	23	14	50	30	19	17	9	5	AUS06.262	Susceptible	Australia	10
20	17	16	42	11	26	15	11	8	AUS06.357	Susceptible	Australia	10
25	18	17	46	20	23	11	10	8	Af293	Susceptible	UK	
24	19	14	51	14	29	10	7	9	IFM 60237	Resistant (P216L)	Japan	5
20	16	25	41	17	23	5	11	10	IFM 59073	Susceptible	Japan	4
25	23	11	0	9	7	7	10	8	1272/09	Susceptible	India	9
20	24	18	6	21	16	11	9	10	IFM 59359	Susceptible	Japan	13
20	16	14	8	17	23	5	11	10	IFM 62351(59355-1)	Susceptible	Japan	5
23	22	16	97	12	7	16	9	10	1962	Resistant (TR_{34}/L98H)	Germany	14