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Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) patients

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Objectives: Patients with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED, APS-I) suffer from chronic candidosis caused mainly by *Candida albicans*, and repeated courses of azole antifungals have led to the development of resistance in the APECED patient population in Finland. The aim of our study was to address whether the patients are persistently colonized with the same or genetically closely related strains, whether epidemic strains are present and which molecular mechanisms account for azole resistance.

Methods: Sets of *C. albicans* ($n=19$) isolates from nine APECED patients reported with decreased susceptibility to fluconazole isolated up to 9 years apart were included. The strains were typed by multilocus sequence typing. *CDR1/2*, *MDR1* and *ERG11* mRNA expression was analysed by northern blotting and Cdr1, Cdr2 and Mdr1 protein expression by western blotting, and *TAC1* and *ERG11* genes were sequenced.

Results: All seven patients with multiple *C. albicans* isolates analysed were persistently colonized with the same or a genetically closely related strain for a mean of 5 years. All patients were colonized with different strains and no epidemic strains were found. The major molecular mechanisms behind the azole resistance were mutations in *TAC1* contributing to overexpression of *CDR1* and *CDR2*. Six new *TAC1* mutations were found, one of which (N740S) is likely to be a gain-of-function mutation. Most isolates were found to have gained multiple *TAC1* and *ERG11* point mutations.

Conclusions: Despite clinically successful treatment leading to relief of symptoms, colonization by *C. albicans* strains is persistent within APECED patients. Microevolution and point mutations occur within strains, leading to the development of azole-resistant isolates.

Keywords: chronic oral candidosis, candidosis, CMC, fluconazole, resistance, TAC1, CDR1, CDR2

Introduction

Autoimmune polyendocrine syndrome type I (APECED, APS-I) is an autosomal recessive disease caused by loss-of-function mutations of the autoimmune regulator gene (*Aire*) on chromosome 21 q22.3.[1,2] Lack of expression of the *Aire* protein allows autoreactive T cells to escape negative selection in the thymus. This leads to an autoimmune reaction mainly against various endocrine glands as well as to the development of high-titre auto-antibodies against interferon-ω and -α.[2] Although the syndrome is rare, cases have been reported in several geographical areas.[3] It has a relatively high prevalence in Finland (1:25 000).

The first symptom of the disease in early childhood is often a superficial *Candida* infection of the oral mucosa.[3] Most patients

develop high titres of *Candida*-specific antibodies but fail to respond to *Candida* antigens and develop chronic mucocutaneous candidosis (CMC).[4–7] Consequently, patients receive repeated courses of antifungals from childhood. In the Finnish APECED population, liberal use of azole antifungals has resulted in clinical and microbiological resistance to azoles of the colonizing *Candida albicans* strains.[8–10] Emergence of cross-resistance between the azoles that the patients had been treated with and newer azole compounds that they had not been exposed to has also been reported.[10] In HIV patients, long-term use of azoles has similarly been reported to cause loss of susceptibility of *C. albicans* to fluconazole as well as the development of azole cross-resistance.[11] The resistance mechanisms operating in *C. albicans* from APECED patients have not yet been investigated.

Several mechanisms are known to result in azole resistance in *C. albicans*. Azoles, including fluconazole, target lanosterol 14α-demethylase, which is a product of the *ERG11* gene.[12] Point mutations and upregulation of *ERG11* can occur and have been identified in resistant clinical isolates.[12–14] The majority of the point mutations associated with resistance alter the binding of azoles to Erg11p.[15] Another mechanism involved in azole resistance is the failure to accumulate azoles in yeast cells, which may be caused either by impaired drug import or increased drug efflux.[16] Multidrug efflux transporters of the ATP-binding cassette (ABC) superfamily and of the major facilitator (MF) class have a key role in this low-level azole accumulation. *CDR1* and *CDR2*, genes for ABC transporters, and *MDR1*, a gene for MF transporters, have been shown to be upregulated in resistant strains, leading to enhanced drug efflux.[12,13,17] This in turn leads to a reduced inhibition of their target encoded by the *ERG11* gene. *MDR1* is often not detectable in azole-susceptible strains, whereas the transcription of *CDR1* may be detectable also in azole-susceptible strains and is elevated in strains with decreased susceptibility. Deletion of *CDR1* and *CDR2* results in hypersusceptible isolates.[13,18] *CDR2* upregulation is usually combined with *CDR1* upregulation due to a mutual activator, the transcriptional activator of *CDR* genes (*TAC1*).[19] *TAC1* is located close to the mating-type locus (*MTL*), and previous studies have shown a correlation between homozygosity (a/a or α/α) at the *MTL* and azole resistance.[14,20,21]

It has been demonstrated that *TAC1* mutations, so-called gain-of-function (GOF) mutations, are associated with hyperactivity of the encoded protein, which is itself responsible for enhanced transcription of *TAC1* target genes, including *CDR1* and *CDR2*.[19] It is therefore expected that isolates with high *CDR1* and *CDR2* expression will exhibit GOF mutations. Mrr1p has been shown to control *MDR1* expression in *C. albicans* and GOF mutations in the *MRR1* gene result in upregulation of *MDR1*.[22]

Using sets of multiple isolates, our study aimed firstly to analyse whether APECED patients with persistent oral candidosis were colonized with the same *C. albicans* strain over several years or whether they had become re-infected with new strains after antifungal treatment. Secondly, we asked whether epidemic strains existed within the APECED population in Finland. Thirdly, we analysed the molecular mechanisms behind azole resistance in these isolates and looked for signs of microvariation in the persisting *C. albicans* strains developing resistance.

Patients and methods

C. albicans isolates

The 19 *C. albicans* isolates used in this study had been isolated from 1995 to 2007 from the oral cavities of nine APECED patients suffering from CMC (1–3 isolates/patient). Patients with at least one isolate with decreased susceptibility to fluconazole or signs of clinical resistance were included. From these patients multiple isolates with various susceptibilities over many years were selected when available. The *C. albicans* isolates had been identified from patient samples using conventional culture and identification methods at the Clinical Microbiology Laboratory of Helsinki University Central Hospital. The identification of *C. albicans* was based on colony morphology on CHROMagar® Candida medium (CHROMagar, Paris, France) and a negative Bichro-Dubli® latex co-agglutination test result for *Candida dubliniensis* (Fumouze Diagnostics, Levallois Perret, France). The strains had been stored in milk–glycerin at −70°C.

Growth media

The *C. albicans* strains were subcultured in complete YEPD broth (1% Bacto peptone; Difco Laboratories, Basel, Switzerland), 0.5% yeast extract (Difco) and 2% glucose (Fluka, Buchs, Switzerland) and subsequently on YEPD agar.

Susceptibility testing

The susceptibility profiles of the isolates to fluconazole and itraconazole were re-analysed by Etest (AB Biodisk, Sweden) according to the manufacturer's instructions and as described previously.[23] Quality control for susceptibility testing was performed using *C. albicans* strains ATCC 90028 and ATCC 24433 and *Candida glabrata* strain ATCC 90030.

Strain typing

Multilocus sequence typing (MLST) was used to type the *C. albicans* isolates. Sequences of bases in the gene fragments from seven housekeeping genes (*AAT1a*, *ACC1*, *ADP1*, *MPI1b*, *SYA1*, *VPS13* and *ZWF1b*) were determined and the isolates were tested by MLST as described earlier using an ABI Prism 3700 genetic analyser (PE Applied Biosystems).[24,25] In brief, isolation of genomic DNA was performed as described earlier from an overnight culture.[26] DNA polymerase Expand High Fidelity (Roche, Basel) was used for the amplification of DNA by PCR. Sequences of primers used are listed in Table 1. Sequence data were analysed for polymorphisms using Contig Express software (Invitrogen, Basel). Sequence data were entered into the MLST database (http://test1.mlst.net) to provide a diploid sequence type (DST) for each strain. According to sequence types, strains were analysed to determine clonal clusters by the eBURST program and each strain was given a clonal cluster number. Clonal clusters were defined as groups of isolates with six of the seven genotype sequences being identical.[27] The unweighted pair-group method with arithmetic averages (UPGMA) was used to display strains as a dendrogram.

Analysis of MTL status

The *MTL* type/status was determined by PCR by amplifying the *MTLa* and *MTLα* genes as described previously.[28] Sequences of primers used are listed in Table 1. The *MTL* was assessed as heterozygous (a/α), homozygous (a/a) or homozygous (α/α). The correlations between the *MTL* type and MIC and between *MTL* type and aggregated years of exposure to azoles were analysed. Aggregated years on azoles signifies the number of years the patient had been on one or more azoles

Table 1. Primers used in this study

Primer	Sequence
MLST	
*AAT1*a-F	5′-CAGCAACATGATTAGCCC-3′
*AAT1*a-R	5′-ACTCAAGCTAGATTTTTGGC-3′
ACC1-F	5′-GCAAGAGAAATTTTAATTCAATG-3′
ACC1-R	5′-TTCATCAACATCATCCAAGTG-3′
ADP1-F	5′-GAGCCAAGTATGAATGATTTG-3′
ADP1-R	5′-TTGATCAACAAACCCGATAAT-3′
*MPI*b-F	5′-ACCAGAAATGGCCATTGC-3′
*MPI*b-R	5′-GCAGCCATGCATTCAATTAT-3′
SYA1-F	5′-AGAAGAATTGTTGCTGTTACTG-3′
SYA1-R	5′-GTTACCTTTACCACCAGCTTT-3′
VPS13-F	5′-TCGTTGAGAGATATTCGACTT-3′
VPS13-R	5′-ACGGATGGATCTCCAGTCC-3′
*ZWF1*b-F	5′-GTTTCATTTGATCCTGAAGC-3′
*ZWF1*b-R	5′-GCCATTGATAAGTACCTGGAT-3′
MTL status	
*MTL*aplha 1-F	5′-TACATTCTGGTCGCGATGCTC-3′
*MTL*aplha 1-R	5′-GTAATCCAAAGCCTCGCATAA-3′
Northern blotting	
CDR1-F	5′-AGTTGAGATCTACCCTTTAAGATATT-3′
CDR1-R	5′-TAATGACCAATTGAATCCCGATTCAG-3′
CDR2-F	5′-TGGTATATAAACTGGACAACATATCTG-3′
CDR2-R	5′-TAATGAACCAATTGAATCCCGATTCAG-3′
*ERG11*N	5′-CGGGATCCATGGGTGGTCAACATACTTCT-3′
*ERG11*C	5′-CGGAATTCCCTGCTGGTTCAGTAGGTAAAAC-3′
*BEN*RN	5′-AAAAGCTTATGCATTACAGATTTTTAAGAG-3′
*BEN*RC	5′-AAAAGCTTCTAATTAGCATACTTAGATCTT-3′
ACT1-RT-PCR-F	5′-GTTCCCAGGTATTGCTGAAC-3′
ACT1-RT-PCR-R	5′-CAATGGATGGACCAGATTCG-3′
TAC1 sequencing	
Amplification	
Zinc2-604	5′-ATAAGAGTGGCATGTGATA-3′
Zinc2-1123	5′′-GATGCCAACGAATTATTGA-3′
Zinc2-1708	5′-CAGAATTCGTTGGAGAATA-3′
Zinc2-2242	5′-GCCTTGTTACAATCAAGAA-3′
Zinc2-2683	5′-GCAGCATATCTTGTATTAG-3′
Zinc2-3224	5′-ATGCTCAGTCACCAAGTTA-3′
Zinc2-3087c	5′-GGTGTTCCTGCTACCACAA-3′
Zinc2-1789c	5′-ACATCAACAATGCCTTCTAC-3′
Zinc2-1247c	5′-TCTTCACCGTATGAACCTA-3′
Zinc2-778c	5′-CGTTGCTATTGGCGGTTGA-3′
Zinc2-1169	5′-TGTTGGTACTCATTCAATT-3′
Zinc2-1722	5′-TTGGAGAATAGTGCCATTT-3′
Zinc2-1510	5′-GCTACCAAGCGAAGGAGAT-3′
Zinc2-2465c	5′-TCTCTCGCCTAATTGACGT-3′
Sequencing	
ZCF36SEQ1	5′-ATTACAATGTGTCCCACACAGG-3′
ZCF36SEQ2	5′-CAGTTTACTTTATCCATTTATGCC-3′
ERG11 sequencing	
Amplification	
ERG11-xho	5′-GCGCAACTCGAGCTCATATGAACAAGGTTGGGTAGTAA-3′
ERG11-Kpn	5′-GAGCATGGGTACCGGCGCGCGATTGTACGT GG-3′
Sequencing	
ERG11-3B	5′-CCCATTAAGAATCCCTGAA-3′
ERG11-5B	5′-CAGGGATTCTTAATGGGT-3′
ERG11-4	5′-CTGCTGGTTCAGTAGGTA-3′
ERG11-6	5′-GAGCAAATGAACGGTCAA-3′

MLST, multilocus sequence typing; *MTL*, mating-type locus.

aggregatively before isolation of the strain. The years of concomitant exposure to multiple azoles have been multiplied by the number of prescribed azoles.

Northern blotting

Small-scale isolation of total RNA was performed from liquid cultures in logarithmic growth phase as described earlier.[26] Northern blotting was performed to determine the mRNA expression for *CDR1*, *CDR2*, *ERG11* and *MDR1* as described previously.[13] RNA samples were separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane using the Vacuum Blotting System (Hoefer Scientific Instruments, San Francisco, CA, USA). Probes were labelled with [α-^{32}P]dATP with random priming using the MegaPrime DNA Labeling System dNTP Kit (GE Healthcare) according to the manufacturer's instructions. Radioactive signals were revealed by exposure to Kodak BioMax MR film (GE Healthcare). Signals obtained in blotted membranes were quantified by counting radioactivity (Typhoon Trio; GE Healthcare). *CDR1*, *CDR2*, *MDR*, *ERG11* and *ACT1* probes were prepared by PCR using primers CYP-CB and CYP-NS2, ACT-RT–PCR-F and -R. Primers used in this study are listed in Table 1. As a control for the evaluation of expression levels of *CDR1/2*, *MDR1* and *ERG11*, the membranes were hybridized with *ACT1* and the amount of RNA was normalized according to the expression of *ACT1*. The azole-susceptible strain isolated in 1995 from Patient 2 (fluconazole MIC 1 mg/L) was chosen as a baseline control strain (0-level) and the expression levels of the other isolates were quantified relative to the susceptible strain.

Immunoblotting

Western blotting was performed to evaluate Cdr1, Cdr2 and Mdr1 protein expression. A *C. albicans* strain (DSY3849) known to overexpress Cdr1p and Cdr2p was used as a positive control. A positive control for Mdr1p was established by exposing the azole-susceptible strain isolated in 1995 from Patient 2 to benomyl for 30 min as described previously.[29] Cell extracts were prepared as described by Sanglard *et al*.[30] Ten microlitres of solubilized yeast protein was separated by 10% SDS/PAGE and transferred by western blot onto a nitrocellulose membrane (Bio-Rad Membrane). Immunodetection of Cdr1, Cdr2 and Mdr1 was performed as described previously.[19] A Super Signal West Pico Chemiluminescent Kit (Thermo Scientific) and Amersham Hyperfilm (GE Healthcare) were used according to the manufacturer's instructions. The protein signals visualized on the exposed films were interpreted as positive or negative.

Sequence analysis of ERG11 and TAC1

Genomic DNA for sequencing for *TAC1* and *ERG11* mutations was extracted as described earlier.[26] Sequences were amplified from genomic DNAs by standard protocols as described previously for *TAC1*[31]

and *ERG11*.[31] The sequences of primers used are listed in Table 1. The alleles were sequenced using an AB3730XL DNA Analyzer (Applied Biosystems). The resulting data were analysed using the Mutation Surveyor software (Soft Genetics).

Statistical analysis

Data were analysed by using Graph Pad Prism version 5.00 (GraphPad Inc., San Diego, CA, USA). Geometric means and range were used for the analyses of MICs. The two-tailed Mann–Whitney *t*-test was used for the comparisons of groups, and Spearman's rho (r_S) was used for the analyses of correlations. Correlations are presented with a 95% confidence interval and *P* value. *P* values of less than 0.05 were considered statistically significant.

Results

MLST typing

All seven patients with multiple *C. albicans* isolates analysed were persistently colonized with the same strain for a mean of 5.0 years (range 1–9) (Table 2). Five of these seven patients (2, 4, 5, 6 and 7) had a pair of isolates identical by MLST. The mean time

Table 2. Characteristics of *C. albicans* isolates used in this study

Patient number/ year of isolation	DST	Clonal cluster	MIC (mg/L)		Time on antifungal (years)		*MTL*	Genes overexpressed	Mutations detected by sequence analysis[a]	
			FLC	ITC	FLC	azoles[b]			*TAC1*	*ERG11*
Patient 1										
2001	1152	3	24	1	6	47	α/a	*CDR1*, *CDR2*	**F973S**, **S290C**	Y132F[14]
2004	1156	3	64	0.5	9	53	α/α	*CDR1*, *CDR2*	**F973S**, **S290C**	Y132F[14]
Patient 2										
1995	360	4	1	0.094	1	12	α/a			**H283R**
2001	360	4	64	12	7	41	a/a	*CDR1*, *CDR2*	A736T[31]	**H283R**
Patient 3										
2006	1157	1	48	1.5	6	36	α/a	*CDR1*, *CDR2*	**N740S**	
Patient 4										
2001	1151	1	48	1	6	22	α/a	*CDR1*, *CDR2*	**N740S**	
2006	1493	1	6	1.5	7	23	a/a	*CDR1*, *CDR2*	N977D[31], **P276L**	
2007	1493	1	32	4	7	24	a/a	*CDR1*, *CDR2*	ND	ND
Patient 5										
1996	1489	S	8	0.75	2	13	α/a	*CDR1*, *CDR2*	N972S[31]	
2001	1489	S	48	12	2	20	α/α	*CDR1*, *CDR2*	N972S[31]	G450E[35], D153E[36]
2004	1318	S	32	1.5	2	29	α/α	*CDR1*, *CDR2*	N972S[31]	G450E[35], D153E[36]
Patient 6										
2004	1494	11	128	2	3	28	a/a	*CDR1*, *CDR2*	E461K[31]	
2005	1494	11	32	1.5	3	28	α/a		E461K[31]	S405F[30]
Patient 7										
1995	1490	4	48	2	4	11	α/a	*CDR1*, *CDR2*	R673Q[31]	V488I[35]
2001	1158	15	48	0.19	7	23	α/a		**S108N**	**H283R**
2004	1490	4	128	2	10	29	α/a	*CDR1*, *CDR2*	R673Q[31]	V488I[35]
Patient 8										
2004	1154	2	48	0.75	3	22	α/a	*CDR1*	H741Y[31]	
2006	1492	2	24	1.5	3	23	α/a		H741Y[31]	
Patient 9										
2004	203	1	96	2	5	15	α/α	*CDR1*, *CDR2*	**N740D**	G464S[14]

MIC, minimum inhibitory concentration; DST, diploid sequence type; FLC, fluconazole; ITC, itraconazole; *MTL*, mating-type locus; ND, sequence not determined; S, singleton.

[a]Mutations A736T, N977D, N972S, E461K, R673Q and H741Y found in *TAC1* alleles have previously been described as gain-of-function (GOF) mutations. Mutations S108N, P276L, S290C, F973S, H283R, N740D and N740S (in bold) have not been previously described.

[b]Aggregated years on azoles before isolation of the strain.

elapsed between the isolation of these strains was 4.4 years (range 1–9). The isolates from the remaining two patients with multiple isolates (Patients 1 and 8) differed in one or two genotypes and were designated by different DST numbers but assigned to the same clonal cluster. These differences mainly resulted from a loss of heterozygosity at the sequenced loci. In one patient (Patient 7) two strains belonging to different clonal clusters were identified. Isolates from three patients (Patients 3, 4 and 9) belonged to clonal cluster 1, and isolates from two patients (Patients 2 and 7) belonged to clonal cluster 4. All other sets of isolates belonged to different clonal clusters (2, 3 and 11). Isolates from Patient 5 were not assignable to a clonal cluster and were designated as singletons. The UPGMA dendrogram of the isolates is shown in Figure 1. Isolates that belonged to clonal cluster 1 but were from different patients did not co-cluster with very high levels of similarity, whereas those that belonged to clonal cluster 4 formed a more compact cluster. However, all patients were colonized with different strains and no epidemic strains were found.

Susceptibility profiles of C. albicans isolates

The MIC of fluconazole for three isolates from three patients (2/1995, 4/2006 and 5/1996) was ≤8 mg/L (Table 2). The remaining 16 isolates had MICs ≥16 mg/L, of which five had MICs ≥64 mg/L. One patient (Patient 2) had one isolate (isolated in 1995) with an MIC of ≤0.125 mg/L of itraconazole and the fluconazole MIC for this isolate was 1 mg/L. All other isolates had MICs of ≥0.25 mg/L of itraconazole, and 14 of these isolates had MICs of ≥1 mg/L. There was an increase in the fluconazole MICs for the sets of isolates from four (Patients 2, 4, 5 and 7) of the remaining five patients, with two isolates identical by MLST. An increase in the itraconazole MICs was seen in the sets of isolates from three (Patients 2, 4 and 5) of these five patients.

One patient set (Patient 1) showed an increase in the MIC of fluconazole only. Four of the five identical DST pairs showed an increase in fluconazole and itraconazole MICs. In all of these cases systemic and/or topical azoles had been used between the isolation of the strains (Table 2). A decrease in the MICs of both azoles could be seen in one set (Patient 6) where azoles had not been used between the isolation of the pair of strains. In another patient set (Patient 8), where only miconazole had been used between the isolation of the strains, a decrease in the MIC of fluconazole but not itraconazole was seen. In neither of these cases, however, did the MICs decrease significantly or fall below the susceptible breakpoints according to the CLSI standard.[32]

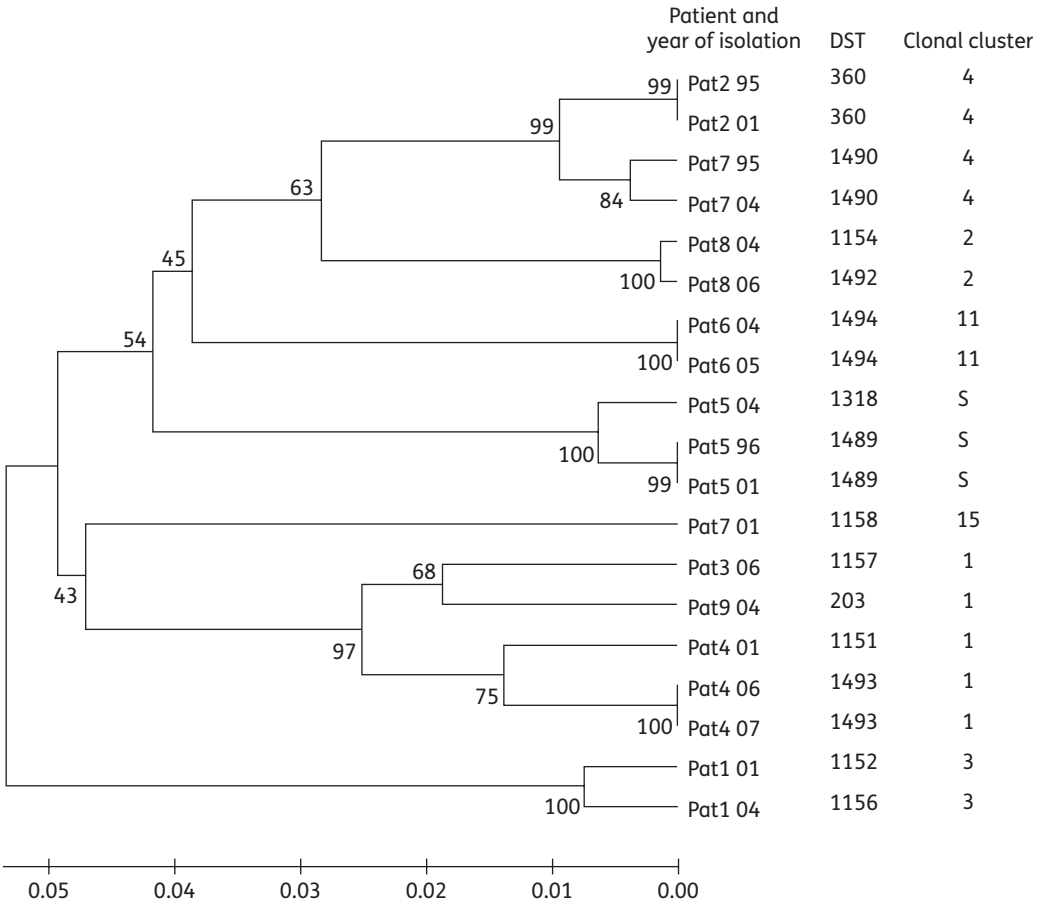

Figure 1. Unweighted pair-group method with arithmetic mean (UPGMA) dendrogram of the isolates typed by multilocus sequence typing (MLST). The scale bar shows genetic distance between the isolates. DST, diploid sequence type; S, singleton.

MTL status

Of the 19 strains, 8 were found to be *MTL* homozygous (a/a or α/α) and 11 were *MTL* heterozygous (a/α). The mean MICs for the homozygous isolates (a/a or α/α) of fluconazole and itraconazole were 45.4 (range 6–128) and 4.7 mg/L (range 0.5–12), respectively. The mean MIC for the a/a type isolates ($n=4$) was 35.1 mg/L (range 6–128) of fluconazole and 4.6 mg/L (range 2–12) of itraconazole. The mean MIC for the α/α type isolates ($n=4$) was 60.0 mg/L of fluconazole and 7.0 mg/L of itraconazole. The heterozygous (a/α) isolates had a mean MIC of 26.7 mg/L (range 1–128) of fluconazole and 1.4 mg/L (range 0.094–2) of itraconazole. The difference between the MICs for homozygous and heterozygous isolates was statistically significant for itraconazole ($P=0.0327$) but not for fluconazole. In the two isolate pairs with identical DSTs where loss of *MTL* heterozygosity could be observed (2/1995 and 2/2001; 5/1996 and 5/2001), the MICs of fluconazole increased. In one patient (Patient 6), who had two strains with identical DSTs isolated 1 year apart, a decrease in the fluconazole MIC was seen concomitantly with a shift from homozygosity to heterozygosity in *MTL* status. *MTL* status did not correlate with years of exposure to azoles. The correlation of *MTL* status with the aggregative exposure to all azoles was also analysed since APECED patients are often prescribed different azole antifungal agents and formulations concomitantly (Table 2). The mean aggregated exposure of the homozygous isolates (a/a and α/α) to azoles was 29.1 years and to fluconazole it was 5.3 years. For the heterozygous isolates, the mean aggregated exposure to azoles was 24.2 years and to fluconazole it was 4.6 years.

CDR1/2, MDR1 and ERG11 mRNA expression

Fifteen (79%) of the 19 isolates showed overexpression of *CDR1* and 14 (74%) showed overexpression of *CDR2* by a mean of 9.8-fold (range 3.5–19.4) and 20.4-fold (range 7.8–65.1), respectively, when compared with the control isolate (Figure 2). Thirteen (87%) of the isolates overexpressing *CDR1* and 12 (86%) overexpressing *CDR2* had fluconazole MICs ≥16 mg/L. Fourteen isolates overexpressed both *CDR1* and *CDR2* and 12 (86%) of these had fluconazole MICs of ≥16 mg/L. All isolates overexpressing *CDR1* or *CDR2* had itraconazole MICs ≥0.25 mg/L. In the four sets of isolates identical by MLST with an increase in fluconazole MICs, the relative expression level of *CDR1* increased by a mean of 3.3-fold (range 0.5–4.4) and that of *CDR2* by a mean of 4.0-fold (range 0.5–11.2). The one pair of isolates identical by MLST (Patient 6), where a decrease in the MIC of fluconazole was seen and the relative expression level of *CDR1* and *CDR2* decreased to the level of the control isolate in 1 year, the patient had not been exposed to any azoles between the isolation of the isolates.

Only two isolates (2006 isolate from Patient 3 and 2004 from Patient 8) showed a slight (3.2- and 4.9-fold, respectively) increase in the relative expression of *MDR1* compared with the control strain. The isolates had an MIC of 48 mg/L of fluconazole and 0.75 and 1.5 mg/L of itraconazole. However, these isolates also showed increased *CDR1* mRNA levels. The expression of *ERG11* was low in our set of isolates (relative expression ranging from 0.2 to 1.3). Fourteen isolates expressed *ERG11* at lower levels than the control, illustrating that azole resistance does not necessarily arise from overexpression of *ERG11*.

Protein expression levels of Cdr1, Cdr2 and Mdr1

All isolates showing elevated Cdr1 and Cdr2 protein levels overexpressed *CDR1* and *CDR2* mRNA. One isolate showed slightly elevated *CDR1* mRNA levels (3.9-fold when compared with the control isolate) but no Cdr1 could be detected (Figure 2). Isolates expressing Cdr1 also expressed Cdr2. Of the isolates that expressed Cdr1 and Cdr2, the mean MIC of fluconazole was 44.5 mg/L and that of itraconazole was 1.8 mg/L, whereas the isolates that did not express Cdr1p and Cdr2p had a mean MIC of 6.9 mg/L of fluconazole and 0.3 mg/L of itraconazole. Mdr1p expression was not detected.

TAC1 and ERG11 sequence analysis

TAC1 mutations were detected in all but one isolate (isolate 1995 from patient 2) (Table 2). Twelve different mutations (A736T, N977D, N972S, E461K, R673Q, H741Y, S108N, P276L, S290C, F973S, N740S and N740D) were found in total. Six of these mutations (S108N, P276L, S290C, F973S, N740S and N740D) have not been described previously. Of these, all but P276L were found in isolates with elevated azole MICs, and N740S occurred in the absence of any other *TAC1* or *ERG11* mutations. N740S was found in two unrelated strains isolated from different patients. The mean relative expression level of *CDR1* and *CDR2* in these two isolates was 11.1-fold (*CDR1*) and 14.6-fold (*CDR2*) compared with the control isolate and their MIC of fluconazole were 48 mg/L.

The sequence analysis of *ERG11* revealed seven point mutations (Y132F, H283R, G450E, D153E, S405F, V488I and G464S) in 11 isolates. Two isolates (2001 and 2004) from Patient 5 had two mutations (G450E and D153E). One mutation (H283R) in *ERG11* had not been previously reported. It was detected in three isolates from two patients (Patients 2 and 7), one of which was susceptible to azoles. In one pair of isolates identical by MLST (Patient 6), acquisition of an S405F mutation was observed in association with a decrease in azole MICs. Pairs of isolates identical by MLST from three patients (Patients 1, 5 and 6) gained additional *TAC1* or *ERG11* mutations as well as maintaining the pre-existing ones.

Discussion

Despite clinically successful antifungal treatment courses, the APECED patients investigated here were found to be persistently colonized with the same *C. albicans* strain over many years. All patients were colonized with different strains and no epidemic strains were found. Our findings are in accordance with those of Li *et al*.,[11] who found three HIV-infected patients to be persistently colonized with closely related *C. albicans* strains despite antifungal treatment. Microvariation was evidenced as small differences between MLST types, resulting in most instances from a loss of heterozygosity at one or more of the sequenced loci. In the present study isolates from APECED patients exhibited similar microvariation.

We have previously shown that the incidence of azole resistance is high in *C. albicans* isolated from APECED patients.[8] In this study patients carrying *C. albicans* with decreased fluconazole susceptibility were included and multiple isolates with various susceptibilities isolated over many years were analysed.

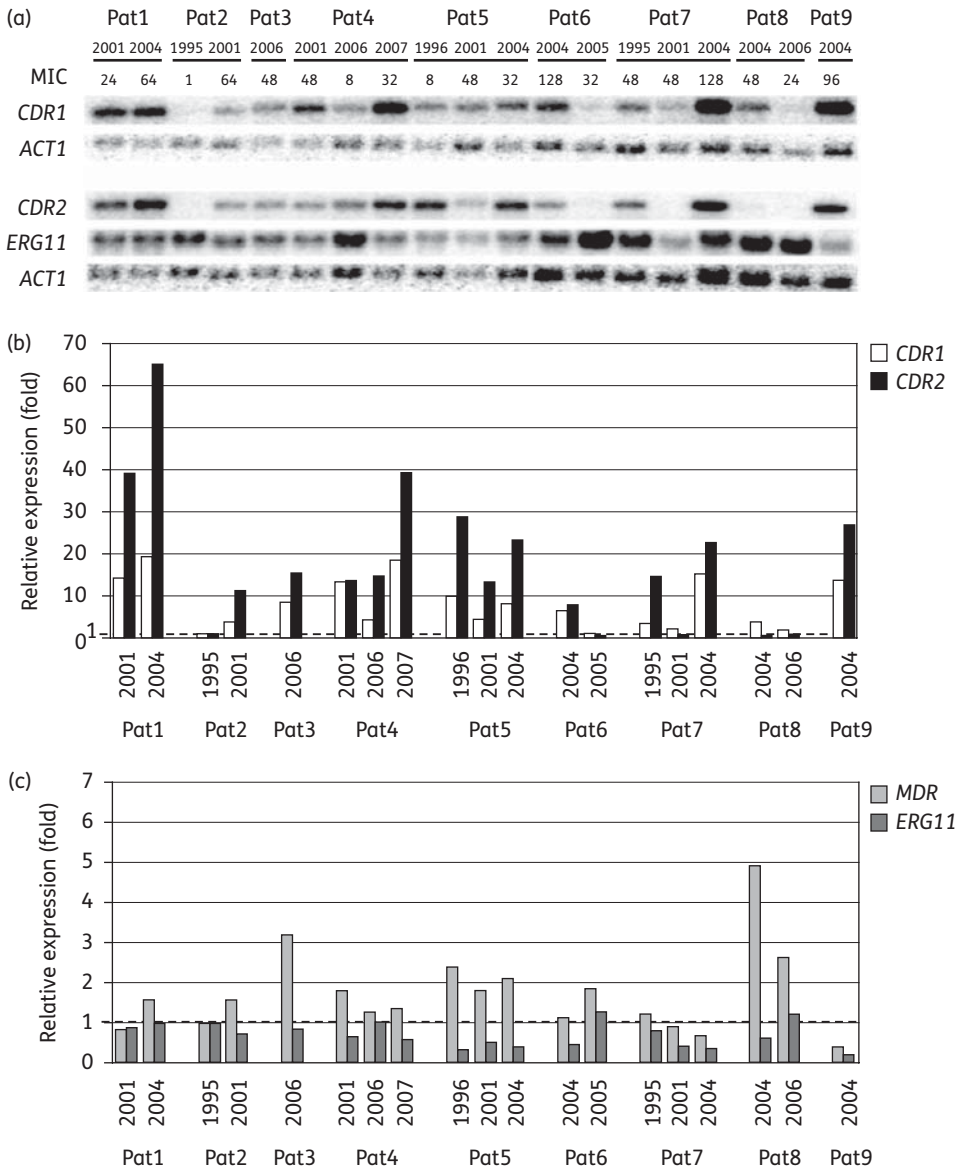

Figure 2. RNA expression levels of *CDR1*, *CDR2*, *MDR1*, *ERG11* and *ACT1*. (a) View of northern blot membranes and MICs (mg/L) of fluconazole. (b) Relative expression of *CDR1* and *CDR2*. (c) Relative expression of *MDR1* and *ERG11*. Expression was quantified by comparison with *ACT1* and then relative expression levels of *CDR1*, *CDR2*, *MDR1* and *ERG11* were calculated for each isolate by comparison with the cognate levels of these genes from the azole-susceptible isolate from Patient 2, isolated in 1995 (fluconazole MIC 1 mg/L). Dotted lines in (b) and (c) represent control isolate 1-fold expression levels for reference.

Of the 19 isolates, 5 were found to be resistant, 11 were susceptible-dose-dependent, and 3 were susceptible to fluconazole.[32] Upregulation of *CDR1* and *CDR2* was significantly associated with the development of resistance. Fourteen (74%) of the isolates showed increased expression of both genes and one isolate only of *CDR1*. The Cdr1 and Cdr2 protein levels correlated with the mRNA levels of *CDR1* and *CDR2*. Upregulation of *CDR1* and *CDR2* was reported to be the main molecular mechanism of resistance in HIV patients with prolonged colonization by *C. albicans* in a study by Perea *et al.*[33] In their study, however, the effect of exposure to antifungal agents was not analysed. We have previously reported that the length of exposure to azoles correlated with azole MICs in *C. albicans* isolated from APECED patients.[34] In the present study, six patients had been exposed to azoles in the intervening years. The relative expression levels of *CDR1* and *CDR2* in closely related pairs of isolates increased on average by 2.1-fold (*CDR1*) and 2.7-fold (*CDR2*). The mean aggregated exposure to azoles between these isolates was 8.0 years.

Several groups have reported a positive correlation between loss of heterozygosity at the *MTL* locus and an increase in azole MICs in *C. albicans.*[14,20,21] In our set of isolates a similar trend was observed, since the MIC of fluconazole increased in both isolate pairs identical by MLST with a loss of heterozygosity

at the *MTL* locus, i.e. there was a correlation between loss of heterozygosity and increased MICs for both fluconazole and itraconazole. A change from homozygosity to heterozygosity at the *MTL* locus was seen in one pair of MLST-identical strains (Patient 6). It is possible that the patient was persistently colonized with a number of closely related strains sharing a parent strain that had undergone microvariation, and the chronology of the isolation of the strains disagrees with the chronology of genetic changes.

It has been demonstrated that GOF mutations in *TAC1* are associated with hyperactivity of the encoded protein, which is itself responsible for enhanced transcription of *TAC1* target genes, including *CDR1* and *CDR2*.[19,35] In this study, *TAC1* mutations were detected in all isolates with decreased susceptibility to azole antifungals. Six new *TAC1* mutations were found in addition to six that had previously been described as GOF mutations.[31] Of the previously unidentified *TAC1* mutations, N740S is a probable GOF mutation since it is the only mutation present in two independent isolates with raised azole MICs. The mutations S108N, S290C, F973S and N740D are to be considered as potential GOF mutations but their role remains unclear because they were detected in isolates that contained other mutations. Six previously described *ERG11* mutations contributing to azole resistance were also identified.[14,16,36–38] In addition, a previously unidentified mutation (H283R) was detected in *ERG11*, but it was found in both azole-resistant and azole-susceptible isolates and is therefore unlikely to be a GOF mutation.

Point mutations in *TAC1* and *ERG11* seemed to remain stable during the period (years) between isolates. Isolates (2001 and 2004) from Patient 5 had acquired two point mutations in *ERG11* after the isolation of the 1996 strain. The mutations were accompanied by loss of heterozygosity at the *MTL* locus, which was also likely to contribute to the increase in the MICs in the presence of a rather surprising 2.2-fold decrease in the expression of both *CDR1* and *CDR2*. However, both *CDR1* and *CDR2* were upregulated in all isolates relative to the susceptible control strain. The *TAC1* mutation remained identical in all these isolates. The patient had not been exposed to fluconazole during the intervening years but had been administered other azoles. Patient 2 had gained a GOF mutation in *TAC1*. This patient had been exposed to fluconazole for 6 years between the isolation timepoints and the MIC of fluconazole had increased from 1 to 64 mg/L. The DST had remained the same. In one pair of isolates identical by MLST (Patient 6), acquisition of a point mutation in *ERG11* (S405F) was detected in association with minor increases in relative *ERG11* and *MDR1* expression and decreases in *CDR1* and *CDR2* expression, resulting in a decrease in azole MICs. In another pair of isolates identical by MLST (1995 and 2004, Patient 7) with identical *TAC1* and *ERG11* mutations and the same *MTL* status, the fluconazole MIC increased (from 48 to 128 mg/L) in association with a 4.3-fold increase in *CDR1* and 1.5-fold increase in *CDR2* expression levels.

In conclusion, our results confirm that APECED patients become persistently colonized with unique *C. albicans* strains undergoing microvariation over time. Clinically successful treatment has not led to eradication of the colonizing *Candida* population and re-infection with another strain. The major molecular mechanisms mediating azole resistance were GOF mutation in *TAC1*, contributing to overexpression of *CDR1* and *CDR2*, and point mutations in *ERG11*. Six new *TAC1* mutations were detected, one of which (N740S) is likely to be a GOF mutation. Most isolates were found to have gained multiple *TAC1* and *ERG11* point mutations. However, the role of individual mutations in azole resistance in *C. albicans* needs further analysis.

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