Summary

*Candida albicans* is a dimorphic organism, which exists both as yeast and hyphal forms. Generally, it exists as a commensal flora of skin, mouth, gastrointestinal tracts and female genital tracts in most healthy individuals. But under condition of immune dysfunction, they can become an opportunistic pathogen causing recurrent chronic oral and vaginal candidiasis as well as life threatening systemic disease. In the recent past *C. albicans* has acquired considerable significance due to their ability to develop resistance against antifungal, such as azoles, in patients undergoing long-term or prophylactic treatment. Mechanism of drug resistance in *C. albicans* have been studied in detail and found to be due to the involvement of three classes of proteins: Erg11p, Major facilitator superfamily (MFS) pumps, and ATP–binding cassette (ABC) pumps. Notably, overexpression of the genes encoding the drug extrusion pumps belonging to the ABC and MFS families is the most common one.

Among ABC transporters, overexpression of the Cdr1p and Cdr2p is the major mechanism that contributes towards azole resistance in *Candida albicans*. This mechanism of survival is certainly not exclusive to this pathogen alone; tumor cells also utilize it in order to resist chemotherapy through overexpression of the Cdr1p homologues, i.e. P-glycoprotein (P-gp)/*MDR1* and the multidrug resistance associated protein (MRP1). An overexpression of P-glycoprotein (P-gp)/human MDR1 remains the most documented and well-characterized example of a drug extrusion pumps that result in the failure of chemotherapy in tumor cells. The presence of proteins homologous to P-gp in all the organisms ranging from the prokaryotes to eukaryotes portrays extrusion of drugs as a general theme of mechanism of MDR.
SUMMARY

Apart from this, several single nucleotide polymorphisms (SNPs) are known to exist in human MDR1/ P-gp and ABCB1 that lead to change in amino acids and subsequently affect the functionality of the protein. However, existence of single nucleotide polymorphism (SNP) in CDR1 ORF (P-gp homologue) or in any other efflux proteins of C. albicans that could affect the substrate specificity and efflux mediated by the protein is not reported. Hence, we were interested in looking towards this aspect.

In order to study allelic variants of ABC drug transporter Cdr1p, the major drug efflux pump protein, in clinical isolates of C. albicans, our first objective was to collect azoles resistant C. albicans from various groups of immunocompromised patients undergoing treatment at major hospitals in Delhi (AIIMS and Safdarjung) either as outdoor or admitted patients. So for this study we collected around 178 candida samples from burn patients and 100 samples from out door patients. All clinical samples were screened for C. albicans by classical as well as by molecular methods. Our identification results showed that 33% of the clinical isolates from burn patient are C. albicans compared to 89% in out door patients. Additional C. albicans strains from our laboratory stock, originally isolated from immunocompromised patients, were also included in the resistotyping to increase the pool of azoles resistant strains. In vitro fluconazole susceptibility of all the C. albicans isolates was determined by micro dilution assay following NCCLS M27-A2 standard protocol. The percentage of resistant isolates were found to be 5.2 % in burn isolates, 4.7 % in HIV/AIDS isolates, 33.3% in diabetic isolates and surprisingly in the out door category not a single isolate was found to be drug resistant.
Since, one of our objectives was to analyze single nucleotide polymorphism (SNP) in CDR1 gene of azoles resistant C. albicans clinical isolates, a few of the drug resistant isolates were randomly chosen from the pool of the drug resistant C. albicans for CDR1 sequence analysis. Two genetically match pairs of C. albicans strains (sensitive and resistant) from our lab repository were also included in the SNP analysis. The drug resistance profiles of these selected isolates were rechecked before studying SNP analysis of the CDR1 gene.

In SNP analysis, we have compared the CDR1 gene sequences of the isolates, which were intrinsically resistant to fluconazole and showed no overexpression of the gene, with the CDR1 sequences of the control resistant isolates that showed over expression of the gene. Sequencing of the CDR1 gene from SC5314 control strain of C. albicans (drug sensitive) did not reveal any SNPs confirming the previous observation that CDR1 is localized within a homozygous region of the chromosome 3. CDR1 gene sequence analyses of the test samples, however, revealed 53 SNPs; out of which six were non-synonymous SNPs (NS-SNPs) implying a change in amino acid. The identified SNPs were found in two or more allelic combinations in different sensitive or resistant isolates. Out of the six NS-SNPs three are new (not reported earlier) namely, E948P, T950S and F1399Y. F1399Y appeared to be a unique and was present in only one of the naturally occurring azole resistant isolates obtained from Indian diabetic patients. However, when the same mutation was introduced into the wild type CDR1 gene of a drug sensitive control isolate, no change in Cdr1p function was observed. This result indicates that a single amino acid substitution may or may not be sufficient to induce significant changes in the function of the gene product.
Taken together, our SNP analyses reveals that the naturally acquired mutation in Indian clinical isolates (intrinsic resistance) in Cdr1p are selective which do not allow the protein to genetically evolve in a manner that could alter its functioning in terms of substrate recognition, specificity and efflux activity. The fact that we did not detect any allelic variation in the conserved regions of the Cdr1p of the isolates, which are presumably critical for protein function, supports our conclusion. Collectively, our findings suggest that NS-SNPs found in either intrinsic or acquired resistant isolates do not affect the functions of the Cdr1p. However, one cannot rule out the possibility of a functional relevance of these NS-SNPs if they occur in a combination that might affect the resistance phenotype. Therefore, it appears that the degree of sequence variation in terms of NS-SNPs is far greater in naturally acquired azoles resistant C. albicans clinical isolates of Cdr1p than the matched pair isolates which have acquired azole resistance due to prolong exposure to the drug.

Overexpression of ABC transporters viz; Cdr1p and Cdr2p is the major mechanism that plays a key role in azole resistance of C. albicans. However, involvement of other ABC transporters in drug resistance has not yet been explored in C. albicans. Though, there are few reports in other organisms which show that other transporters of ABC family are involved in drug resistance. For example Snq2p of S. cerevisiae belongs to ABC family and has been shown to confer resistance to 4-nitroquinoline-N-oxide, sulphomethuron methyl, o-phenanthroline etc. when over expressed. Azole-resistant Candida glabrata isolates with unaltered expression of CgCDR1/ CgCDR2, but with upregulated CgSNQ2 have also been identified. Decreased azole resistance by disruption of CgSNQ2 has also been reported, whereas reintroduction of the gene fully reversed the effect.
In order to test involvement of other ABC transporters in drug resistance in *C. albicans*, we have cloned *CaSNQ2*, expressed in heterologous host *S. cerevisiae* and characterized its drug resistance property. It is known that the pattern of codon usage varies among species; in fact some of the 'preferred' codons in *C. albicans* differ from those in *S. cerevisiae*. In silico analysis of *CaSNQ2* gene sequence reveals one such codon ‘CTG’ at five different positions, which codes for serine in *C. albicans*, where as it normally codes for leucine in yeast. Since we had to use a heterologous system (*S. cerevisiae*) for the expression of the *CaSNQ2*, we replaced all the ‘CTG’ by ‘TCG’ so that it codes serine in that background. When functionality of the CaSnq2p was checked using various drugs we observed resistance to 4-nitroquinolineoxide, terbinafine and 1, 10 o-phenathroline. CaSnq2p, however, did not exhibit resistance to cycloheximide, miconazole, fluconazole, rhodamine 6G (R6G), ketoconazole and itraconazole in contrast to the CaCdr1p, suggesting that azoles are not the preferred substrate of CaSnq2p. Similarly, nystatin and Rhodamine 6G (R6G) were also found not to be a substrate of CaSnq2p.

As far as codon biasness is concerned, no significant effect was observed in the broth microdilution resistotyping assay. Nonetheless, in spot assay for resistotyping effect of correct codon usage had been observed; for example ADSNQ2-828 construct (without codon bias) showed relatively higher susceptibility to miconazole and resistance to 4-NQO and terbinafine as compared to ADSNQ2 (with codon bias).

The effect of steroid, oxidative stress and antifungals on the regulation of *CaSNQ2* gene has also been studied. We observed that only 4-NQO significantly enhanced (3.8 fold) the transcription level of *SNQ2*. However, no considerable induction of the gene by β-estradiol, progesterone, oxidative stress (*H₂O₂*) and fluphenazine was observed. The
increase in drug resistance level towards 4-NQO could be attributed to the elevated transcript level of CaSNQ-2.

Taken together, we have shown that although Snq2p and Cdr1p belong to the same ABC superfamily of transporters of \textit{C. albicans}, the proteins mediate resistance to non-identical distinct subset of drugs. Cdr1p mediates resistance to cycloheximide, miconazole, fluconazole, rhodamine 6G (R6G), ketoconazole and itraconazole whereas Snq2p mediates resistance to 4-Nitroquinoline oxide, terbinafine and o-phenanthroline. Interestingly, this is in agreement with the previous observation where \textit{PDR5} and \textit{SNQ2} of \textit{S. cerevisiae} were also shown to be involved in multidrug resistance but the resistance spectra associated with these genes was different. Resistance to cycloheximide is \textit{PDR5}-specific but resistance to 4-NQO is \textit{SNQ2}-specific; whereas as resistance to staurosporine and fluphenazine is attributable to both the genes.