Regulation of Multidrug Resistance Genes of *Candida Albicans*

Scholor
Naseem Akhtar Gaur

Supervisor
Dr. Qazi Mohd. Rizwanul Haq
Department of Biosciences
Jamia Millia Islamia
Co-Supervisor
Prof. Rajendra Prasad
J. N. U.

Candida albicans is an opportunistic diploid fungus that causes superficial as well as systemic infections in immunocompromised and debilitated patients. Widespread and prolonged usage of antifungals, in recent years has led to the rapid development of clinical isolates of *Candida,* which display Multi-Drug Resistance (MDR). Various mechanisms, which contribute towards the development of MDR have been implicated and some of these include, overexpression or mutations in the target enzyme of azoles i.e. lanosterol 14a -demethylase and overexpression of the drug efflux pumps *CDR1* and *CDR2* belonging to the ABC (ATP-Binding Cassette) and *CaMDR1* belonging to MFS (Major Facilitator Superfamily) family of transporters. Among the ABC transporters, high level of expression of *CDR1* invariably contributes to an increased efflux of fluconazole and thus corroborates its direct involvement in drug efflux. While several studies have confirmed that these drug extrusion pump encoding genes are upregulated in azole resistant clinical or laboratory adapted isolates of *C. albicans*, the regulatory mechanisms controlling their expression are yet to be elucidated.

In this study we have examined basal expression of *CDR1* proximal promoter by employing *Renilla* luciferase reporter system. We observed that upon sequential deletion of the proximal promoter, there was modulation in the basal reporter activity. The reporter activity was highest (2.3 fold) in NGY261 (-261 bp from TSP), and was reduced upon subsequent deletions. *DNaseI* foot printing revealed four protected regions (W1, W2, W3 and W4) in proximal promoter which could represent possible trans-acting factor binding sites and thus might be involved in *CDR1* expression. Site directed mutational analysis of three of these protected regions did not significantly affect the basal reporter activity, however, the mutation of W1 led to a considerable enhancement in reporter activity (~ 4 folds) (Figure 1) and was designated as Negative Regulatory Element (NRE).

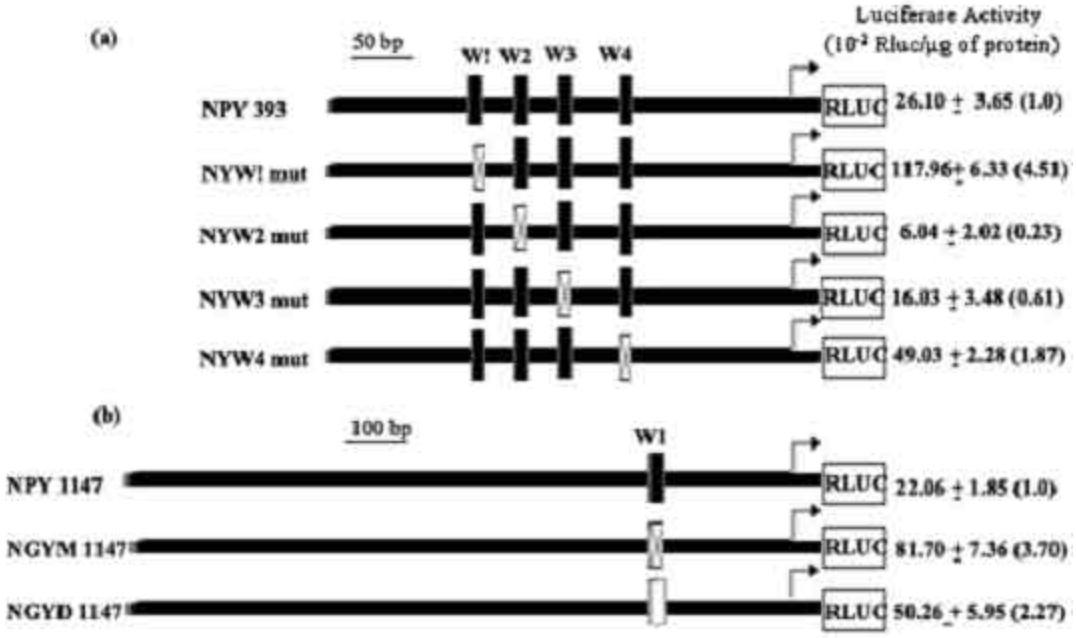

Figure 1. Effect of mutations on the basal *CDR1*-Luciferase activity. a. Schematic representation of *C. albicans* transformants depicting W1-4 mutated 5' *CDR1*-Rluc chimeric constructs. Left to the black bars, constructs names are given where in number indicates the length of the promoter from the transcription start point (TSP). The relative luciferase reporter activity (10^{-2} Rlu m g^{-1} of protein) and fold changes in reporter activities (in parentheses), taking that of the proximal promoter (-393 bp) as 1, are given on the extreme right side of the black bars. The filled boxes (■) indicate the position of the DNaseI protected regions and the crossed boxes (x) represents the mutation of the respective DNaseI protected region. b. The relative luciferase reporter activity (10^{-2} Rlu m g^{-1} of protein) and fold change in reporter activity (in parentheses) after mutation (NGYM1147) or deletion (NGYD1147) of W1 in native promoter (NPY1147) are given on the extreme right side of the black bars. The filled box (■) indicate the position of the DNaseI protected region, the crossed (x) and open box (□) represents the mutation and deletion of the W1 sequence, respectively. The value of reporter activity of single copy integrated vector with out promoter (NGYpCRW3) was always below 0.5 (10^{-2} Rluc m g^{-1} protein) and subtracted from the reporter activity of each transformants. The data shown above are the average +/- standard deviations from three independent experiments with duplicate measurements with two independent clones.

The mutation as well as deletion of W1 sequence in the native promoter (-1147 bp from TSP) and sequential deletion of 5' flanking region-harboring W1 (NRE) also resulted in enhanced promoter reporter activity. When the reporter activity of native (NPY1147) and NRE mutated

(NGYM1147) promoter integrants was monitored throughout the growth phase of *C. albicans*, there was modulation in reporter activity in both the integrants but interestingly, the level of basal reporter activity of the NRE mutated promoter was always ~3 fold higher than the native promoter. UV cross-linking and affinity purification confirmed that a purified ~55 kDa nuclear protein specifically interacts with the NRE. The purified 55.0 kDa NRE binding protein was used for mass spectroscopic analysis and identified as Translation Elongation Factor (TEF1). It was also observed that nuclear proteins isolated from various morphological pathways (*Efg1*, *SKO20*, *RAS1*, *CPH1* and *HSP90*) disrupted strains retained the NRE binding activities in EMSA. On the other hand, complete disappearance of NRE binding activity in EMSA with nuclear extract isolated from *Tup1* knock out strain of *C. albicans* strongly implicated its role in *CDR1* expression via NRE. Deletion of *Tup1* resulted in constitutive hyphal formation of *C. albicans* cells and it has also been demonstrated that *Tup1* act as a global repressor of transcription which either directly interacts to the downstream targets or in association with other factors. Differential expression of *CDR1* has also been demonstrated in transcription profiling of *Tup1* knock out strain of *C. albicans*. It should be noted that mutation or deletion of NRE in *CDR1* promoter resulted in constitutive upregulation of *CDR1*. In order to establish the nucleotide composition of the NRE in detail, we analyzed the flanking sequence harboring negative regulatory element *In-Silico* by using TRANSFEC program. Interestingly NRE along with the flanking sequence was turned out to be a composite element of multiple *cis*-acting regulatory sequences

We have also studied the molecular mechanisms involved in the expression of *CDR1* and the contribution of the identified NRE in expression of *CDR1* gene in fluconazole susceptible and resistant isolates. For this, we selected two pairs of genetically matched fluconazole susceptible and resistant isolates: GU4 (S)/GU5(R) and DSY294(S) /DSY296(R)) of *C. albicans* collected from AIDS patients, wherein *CDR1* is overexpressed in resistant isolates. Mutation in promoter sequences, mutation or alteration in transregulatory factors and changes in mRNA stability and processing are the known mechanisms for the maintenance of constitutive high level of mRNA, which results in the overproduction of the target protein. Mutation(s) in the trans-regulatory factor(s) responsible for the *CDR1* upregulation was confirmed, when *CDR1* promoter cloned from the fluconazole susceptible isolate (SC5314) constitutively overexpressed the *LACZ* and *GFP* reporter genes after integration into the genome of fluconazole resistant isolates (GU5 and DSY296) at *ACT1* Figure 2) and *CDR1* loci. However *CDR1* promoter cloned from the fluconazole resistant isolate was not able to overexpresses the *LACZ* and *GFP* reporter genes after integration into the genome of fluconazole susceptible isolates (GU4 and DSY294). This exchange of promoter between fluconazole resistant and sensitive isolates ruled out the possible involvement of *cis*-acting mutations in *CDR1* upregulation, which was further, confirmed by sequence analysis of the *CDR1* promoter cloned from fluconazole resistant (GU5) and sensitive (GU4) isolates. However pair-wise sequence alignment of *CDR1* promoter cloned either from GU5 or GU4 with SC5314 promoter sequence revealed the existence of two different alleles of the *CDR1* promoter in different isolates.

(a)

(b)

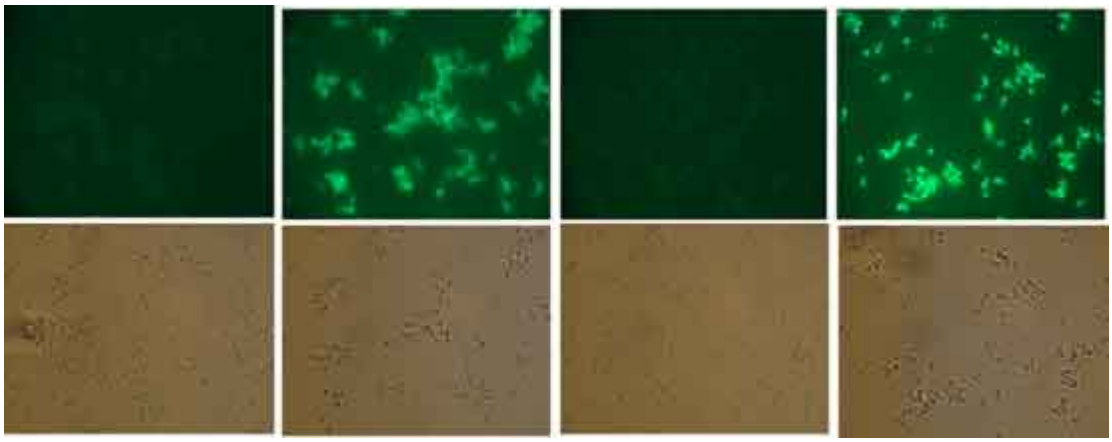

Figure 2. *In vivo GFP* expression driven by *CDR1* promoter at *ACT1* locus of fluconazole susceptible (GU4 and DSY294) and fluconazole resistant isolate (GU5 and DSY296). a Schematic representation of *CDR1P-GFP* reporter construct integrated into the genome of fluconazole susceptible (GU4 and DSY294) and fluconazole resistant isolate (GU5 and DSY296) at *ACT1* locus. b Phase contrast (below) and corresponding fluorescence (above) micrographs of transformants harboring the chromosomally integrated *CDR1P-GFP* fusion are shown in the figure.

In order to rule out the possible involvement of mRNA stability in *CDR1* upregulation, GFP was fused downstream to the *CDR1* ORF in such a way that it was expressed along with the native *CDR1* transcript and the *GFP* fluorescence was examined, a remarkable difference in fluorescence intensity was seen between resistant (GU5) and sensitive (GU4) isolates which was much more distinct as compared to the difference observed by fusion of *GFP* directly downstream to the promoter. A distinct difference in the *GFP* fluorescence was also observed even in the same isolate (GU4) when compared between GU4G1 (GFP fused downstream to the promoter) and GU4G2 (GFP fused down stream to the ORF). In another pair of clinical isolates (DSY294 and DSY296) the difference in fluorescence intensity between resistant and sensitive isolates was comparable in both the cases i.e. after fusion of the GFP downstream to the promoter and to the ORF. However when *CDR1* expression was compared between DSY294G1 (*GFP* fused to promoter) and DSY296G2 (*GFP* fused to ORF) it was observed that after fusion of the *GFP* to the ORF the fluorescence intensity was slightly higher. Therefor it was concluded that along with the mutation in the trans-regulatory factors, mRNA stability also contributes for the maintenance of the constitutive high level of *CDR1* transcript in fluconazole resistant cells. The membrane localization of the Cdr1p in all tested strains was further confirmed by confocal microscopy.

Taken together, *CDR1* promoter is a cluster of multiple *cis*-acting regulatory elements. A negative regulatory element was identified in the proximal *CDR1* promoter and the functionality of NRE was further tested in clinical isolates. The NRE binding protein was identified as TEF1 by MS. Multiple mechanisms (mutation in trans-regulatory factors and modification of mRNA during processing etc.) was found to be contributing to the maintenance of the high level of *CDR1* expression in fluconazole resistant isolates.