# AZOLE HYPERSENSITIVE HYPOTHETICAL PROTEIN UPREGULATE THE INTERMEDIARY METABOLISM AND ERGOSTEROL REGULATORY GENES IN FUNGI

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***ABSTRACT:*** *Study have been focused on azole hypersensitive hypothetical gene NCU01898 in Neurospora crassa. The ketoconazole responding ergosterol gene transcriptional levels have been examined from up regulated and down regulated paterns of ergosterol regulating genes in fungal sterol biosynthesis.Which revealed up regulation in ERG 11 ERG 2, ERG5 as well as ERG6 up regulation under ketoconazole stress gene in NCU01898 as compare to the wild type. However, in the ketoconazole responding ergosterol down regulating genes. ERG7, ERG8 and ERG13 revealed significant differnce in NCU01898 as compare to wild type. However under azole stress along with the upregulation of the ergosterol genes intermediary metabolic pathway specifically carbohydrate metabolism related genes are significantly involved in ketoconazole hyper sensitive mutant NCU01898. Study also revealed higher number of the up regulated gene transcriptional levels in the membrane specific genes. That means NCU01898 shown hypersensitivity due to the intermediary metabolic genes involved in carbohydrates and membrane specific functions. Where as in NCU01898 ergosterol biosynthesis ERG11, ERG2, ERG5 and ERG 6 was upregulated that induced ketoconazole sensitivity in the hypothetical (NCU01898) gene mutant.*

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**Keywords:** Ergosterol biosynthesis, intermediary metabolic genes, transcriptional levels, hypothetical proein, azole sensitive.

## 1. INTRODUCTION

Pathogenic fungi induce pathogenesis among several organisms by regulating various genes. The CDR1 and CDR2 in Candida albican are upregulated under azole stress due to the less drug penetration into the cell. However, deletion of the CaCdr1 and Mdr revealed C. albicansis susceptible to azole [1, 2]. Azole resistance induced by increasing use of FLC causes cross resistance to other azole (Itraconazole, Ketoconazole and Voriconazole)[3]. As in candida glabrata there is additional evidence of Mdr upregulations and the in vitro exposure of floconazole to c.glabrata that induced the erg11 expressions in cross resistance [4]. Aspergillus Species known as a primary pathogen in filamentous fungi is also associated with high morbidity due to drug resistance [5, 6]. The emergence of drug resistance among pathogenic fungi is considered an evolutionary step [7]. Additionally in aspergillus species, the occurrence of multidrug resistance is usually caused by the reduced accumulations of the sterol intermediates that correlate with increased expression of the MDR genes of ABC and the MFS super family [8, 9 and 10]. Aspergillous spp encodes number of ABC family transporters among fungi and yeast, while the clinical isolates evidences are very less [11]. However, there are some further links of the Afu Cyp51AP Knockout which is responsible for innate immunity to FLC and KTC point mutation in AFU Cyp51Ap is major cause of resistance [12]. In fumigatus clinical isolates, azole resistant isolates are associated to be involved in efflux pump over expression. As well as in Apergillus nidulans ortholog AtrA, AtrB transporter of pdr family clones demonstrated fivefold higher level of mRNA in itraconazole exposure,though the Pdr family member AbcA deletion has not shown more sensitivity in A. fumigatous to antifungals [13,14]. However, C.Krussei revealed sensitivity to several azoles such as, voriconazole and posaconazole that is attributed to efflux pumps and ERG11 gene.

Among Cryptococcus species,C.neoformance and C.gatti are commonly found as human pathogens. Where, cryptococcus species is well known in obtaining resistance to increased use of FLC and ITC that is also associated with erg11 mutation and drug efflux pumps up regulation as mentioned in previous studies [15]. C.neoformance Af1p and C.neoformance MdrP are two main efflux pumps. C.neoformance Mdr1p is comprised of (TMP-NBD) topology having higher homology with AflMDr1, Afu MDR1 and ABcB1However there several studies reported in pathogenic fungi and yeast, that ABC transporters pumps azoles out cells and cause resistance [16,17,18]. That further demand to focus on the azole transporters and their homologous conserved protein regulations to gain the broader perspective of pathogenicity among pathogens for better antifungal management.

## 2. MATERIALS AND METHODS

### SAMPLE COLLECTION

Neurospora crassa mutant and the wild type strains were inoculated on Vogel plates. The mycellial disc of 1cm diameter mate was taken and suspended in the 100ml Vogel's liquid media and kept at 28°C with 200rpm incubator for 12 hours. To analyze the transcriptional responses of Ketoconazole in mutants and the wild type Neurospora crassa, 2.5µg Ketoconazole was added into the flasks after 13.5 hours while DMSO was added as control and further incubated at 28hours 200rpm for next 24 hour.

### RNA Extraction

Fungal mycelia taken into the 1.5 ml eppendorff tubes and iced up by liquid nitrogen. RNA extraction has been done conventionally using TRIzol reagent, chloroform after mixing with slight oscillations at room temperature. The collected supernatant was added with 400µl of isopropyl alcohol and centrifuged at 12000rpm for 15 minutes at 4°C. The supernatant was discarded and the tube was washed repeatedly with 70% alcohol. The RNA pellet was diluted 40µl RNA free water while keeping on ice. The concentration of RNA at was measured at OD 260/280 within the range of 1.9-2.1 absorbance and direct to the sequencing.

## RESULTS.

### Drug sensitivity

Among filamentous fungi *Neurospora crassa* have availability of easily accessible genome database and is frequently used as a model organism for studying metabolic mechanisms. Hypothetical protein NCU1898 have no conserved domain known consisted of 425 amino acids. Gene have 6 exons and 2 transcript. Mutant have been tested for antifungal sensitivity test and found hypothetical protein hypersensitive in ketoconazole stress as compare to wild type figure 1.

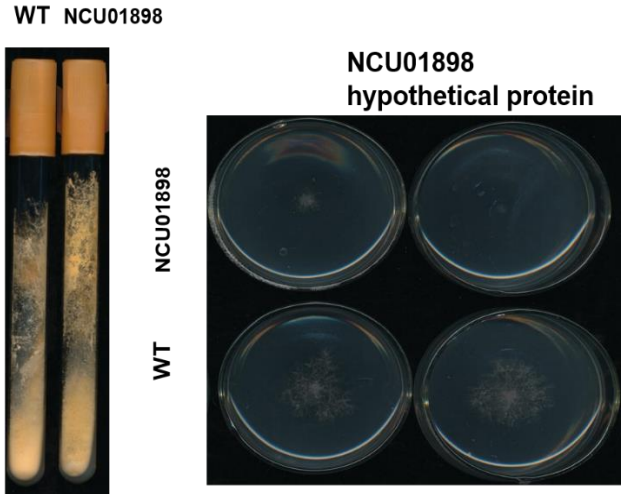


**Figure 1. NCU01898 hypothetical protein revealed hypersensitivity in ketoconazole stress.**

## RNA sequencing of Ergosterol genes

The existing trends of resistance among current antifungals demand study more related genetic aspects of antifungal resistance. Mutants diminished in particular derivatives of the ergosterol biosynthesis pathway are more potent to uncover the under lying phenomena of various genes. However the antifungal azole, commonly interact with sterol demethylase and catalyze the oxidative demethylation of C-14 of lanosterol derivatives of sterol. The ketoconazole responding ergosterol gene transcriptional levels have been examined from up regulated and down regulated paterns of ergosterol regulating genes.Which revealed 358 fold up regulation in ERG 11 gene in NCU01898 as compare to the wild type. Where as ERG 2 with 139.55 fold ERG5 with 9.4 fold where as ERG6 with 28 fold up regulation in ketoconazole stress as compare to the wild type as mention in table 2. However, in the ketoconazole responding ergosterol down regulating genes. Where ERG7 revealed 12.56 fold difference as compare to wild type. ERG8 revealed 26.52 fold change while ERG13 shown significant differnce of 237 fold in NCU01898 (ketoconazole hypersensitive mutant) as compare to the wild type as mention in table 2.

**Table .2. Ergosterol regulating genes in sterol biosynthesis pathway in ketoconazole stress.**

| Up Regulation | GENES | TPM-WT | TPM-WT-KTC | TPM-without KTC | TPM--KTC |
|---|---|---|---|---|---|
| | ERG11 | 69.08 | 676.66 | 85.31 | 317.97 |
| | ERG2 | 26.77 | 296.35 | 129.49 | 156.77 |
| | ERG5 | 83.47 | 174.62 | 113.86 | 184.03 |
| | ERG6 | 19.28 | 174.62 | 46.9 | 146.54 |
| Down Regulation | ERG7 | 23.03 | 4.48 | 12.24 | 17.04 |
| | ERG 8 | 26.48 | 10.63 | 25.15 | 37.15 |
| | ERG13 | 227.38 | 34.98 | 260 | 272.3 |

## 3. Fungal intermediary metabolic genes

Ergosterol, biosynthesis is critical for the fungal membrane fluidity, stability and survival under stress conditions. As well as, a target pathway for antifungals drugs [19]. However under azole stress along with the upregulation of the ergosterol genes intermediary metabolic pathway of the genes in fundamental biomolecules are also involved in ketoconazole hyper sensitive mutant NCU01898. Study revealed higher number of the up regulated gene transcriptional levels in the carbohydrates and membrane specific genes with 23 and 21 respectively. Whereas, ketoconazole upregulated gene number was least in the DNA repair mechanism with 11 genes as mentioned in Figure 2.

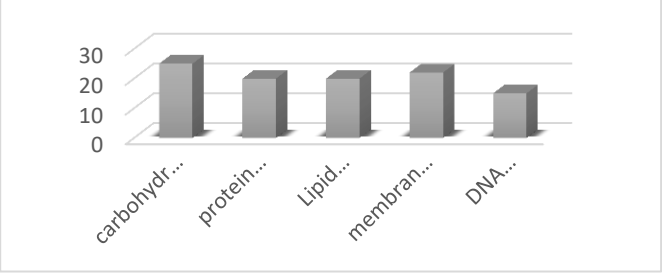


**Figure 2. Differentially activated intermediary metabolic genes of *Neurospora crassa* under azole stress. (Number of genes are presented in vertical pattern).**

## 4. DISCUSSION

Eukaryotes like filamentous fungi and yeast are most frequently available organisms to investigate the basic metabolic mechanisms of cell [21, 22]. Compared to the higher eukaryotic organisms, yeast is more conveniently cultivated on simple media in less time and the accessibility towards the genetic database is widely available. Therefore, it is extensively applicable to study the molecular mechanism of

the diseases [23, 23]. During last two decades multiple fungal species have been applied as model organisms as *N.crassa.* It is an excellent model with 70% available deletion mutants to study the various cellular regulatory pathways. There has been a significant increase in the invasive mycoses with increasing resistance from last decade. The emerging problems steadily with rise of fatal disseminated fungal infections. That prompted to reinvestigate the principal molecular mechanisms. In the past decade azole antifungals have played vital roles in controlling agriculture and human mycoses but with increased resistance to available antifungal worldwide. This also has been observed that fungal species are the part of etiology of various infections due to the increased resistance to available antifungals. That is complicating the infection management. The second major concern is the mode of action of current antifungals which is limited to the toxicity of the in host cell that results in sever side effect among host. Therefore the investigation of wider aspect targets is among the active field.

## 5. CONCLUSION.

Study have been done on a ketoconazole hypersensitive hypothetical gene NCU01898 in *Neurospora crassa*. The ketoconazole responding ergosterol gene transcriptional levels have been examined from up regulated and down regulated paterns of ergosterol regulating genes in fungal sterol biosynthesis.Which revealed 358 fold up regulation in ERG 11 gene in NCU01898. ERG 2 with 139.55 fold, ERG5 with 9.4 fold where as ERG6 with 28 fold up regulation in ketoconazole stress as compare to the wild type. However, in the ketoconazole responding ergosterol down regulating genes. ERG7 revealed 12.56 fold difference as compare to wild type. ERG8 revealed 26.52 fold change while ERG13 shown significant differnce of 237 fold in NCU01898 (ketoconazole hypersensitive mutant) as compare to the wild type. However under azole stress along with the upregulation of the ergosterol genes intermediary metabolic pathway of the genes are also involved in ketoconazole hyper sensitive mutant NCU01898. Study revealed higher number of the up regulated gene transcriptional levels in the carbohydrates and membrane specific genes. That means NCU01898 shown hypersensitivity due to the intermediary metabolic genes involved in carbohydrates and membrane specific functions. Where as in ergosterol biosynthesis ERG11, ERG2, ERG5 and ERG 6 was upregulated that induced ketoconazole sensitivity in the NCU01898 gene mutant.

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