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EXPRESSED SEQUENCE TAGS OF *ASPERGILLUS FUMIGATUS*: EXTENSION OF CATALOGUE AND THEIR EVALUATION AS PUTATIVE DRUG TARGETS AND/OR DIAGNOSTIC MARKERS

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ABSTRACT

Aspergillus fumigatus a fungal pathogen is implicated in a spectrum of allergic and invasive disorders in humans. Validation of transcriptome of pathogen is essential for understanding its virulence mechanism and to identify new therapeutic targets/diagnostic markers. In order to rapidly identify genes of Aspergillus fumigatus we adopted sequencing of cDNA clones. Our earlier effort has lead to identification of 68 expressed sequence tags of Aspergillus fumigatus. Present study describes 52 more expressed sequence tags generated by sequencing 200 phage clones of a non-normalized cDNA library. One of the cDNA clones comprised of the complete coding region for tetratricopeptide repeat domain protein gene. Various homology search algorithms were employed to assign functions to expressed sequence tags coding for hypothetical proteins, and relevance of these expressed sequence tags or their protein products as drug targets/diagnostic markers was examined by searching for homologues in fungi and human.

KEY WORDS

Aspergillus fumigatus, Expressed sequence tags, Tetratricopeptide repeat domain, Drug target, Diagnosis.

INTRODUCTION

Aspergillus fumigatus is an important opportunistic pathogen of humans. The genome of *A. fumigatus* Af293 was sequenced by the whole genome random sequencing method augmented by optical mapping (1). Af293 contains eight chromosomes ranging in size from 1.8-4.9 Mb, for a total of 29.4 Mb of genomic sequence and 49.9% G+C. There are 9,926 predicted protein-coding genes with a mean gene length of 1,431 bp (2).

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Till date most of the transcriptome of *A. fumigatus* has been predicted on the bioinformatic basis and about one-third of the predicted genes are of unknown function, process of annotation of *A. fumigatus* genes needs to be geared up. ESTs (partial cDNA sequences of usually 200 to 700 nucleotides) have demonstrated their worth in the selection of apparently unannotated proteins and putative small peptides from *Arabidopsis* (3,4). This EST and cDNA approach has also been used to annotate the UTRs of genes, to correct the boundaries of introns and exons, and to identify new introns (especially within the UTRs) and probable micro-exons. ESTs have also been used to discover non-canonical splice sites (3,5). Re-annotation of the Arabidopsis genome using a new collection of full-length cDNAs characterized 240 genes that had escaped annotation using the standard gene modelling algorithms (5). The annotations are homology based and EST sequences or clusters inherit the annotative attributes of their match (3).

We undertook sequencing of cDNA clones of *A. fumigatus* from its cDNA library. Efforts have been made to sequence

clones from a normalized cDNA library of *A. fumigatus* (Kessler et al. 2002). However, the sequence data of the 1500 ESTs, reported in their study, is not available in the public domain. Earlier we have presented an account of 68 ESTs generated by sequencing cDNA clones of *A. fumigatus* (6) and the sequence data has been made available on NCBI database (http://www.ncbi.nlm.nih.gov/) for these ESTs.

With a view to extend the catalogue of expressed sequence tags (ESTs) of *A. fumigatus,* we sequenced clones from its non-normalized cDNA library and comparative analyses of these ESTs with human and other fungal protein databases have been carried out.

MATERIALS AND METHODS

Isolation and amplification of cDNA clones from cDNA library: A cDNA library made from mycelial extract of *A. fumigatus*, grown at 37°C was obtained from Stratagene (La Jolla, CA). The library was expanded in XL-1 Blue MRF *E. coli* cells and the Uni-ZAP XR lambda cDNA clones obtained from individual phage plaques were converted to pBluscript SK(+) phagemids by in vivo excision with the help of helper phages as described by the manufacturer. The *E. coli* SOLR cells were infected with the phagemid and were plated onto LB-ampicillin agar plates and incubated at 37°C overnight.

Colony PCR and sequencing of PCR product: Colonies appearing on the plate containing the phagemid having the cDNA insert were used for colony PCR using T3 (5' AATTAACCCTCACTAAAGGG 3') and T7 (5' GTAATACGACTCACTATAGGGC 3') primers. PCR cycling conditions were 94°C / 4 min and 28 cycles of 94°C/ 1 min, 58°C/ 1.5 min, 72°C / 2 min, followed by a terminal extension cycle at 72°C / 7 min. PCR amplification product was purified for sequencing with GFXTM PCR and Gel band purification kit (Amersham Pharmacia Biotech Inc.). Automated DNA sequencing was performed twice using fluorescent dye-terminator chemistry and T3 primer by ABI 377 DNA Sequencer (Applied Biosystems).

Expressed sequence tag (EST) data analysis: To validate the sequences of ESTs obtained, the sequences were subjected to BlastN against the *A. fumigatus* whole genome shotgun assembly at Sanger institute (http://www.sanger.ac.uk/projects/A_fumigatus). ESTs were assigned a putative function based on the match with highest sequence similarity, using the BlastX against National centre for Biotechnology information (NCBI, http://www.ncbi.nlm.nih.gov/) non-redundant protein database of fungi. Sequences which couldn't be assigned a putative function using BlastX were assigned a functional category as per the yeast functional classification catalogue developed by Munich Information Centre for protein sequences (http://www.mips.gsf.de/fungi). Sequence similarity of *A. fumigatus* ESTs with human or other fungi counterparts was identified by using BlastX with the protein databases of *Homo sapiens, Aspergillus fumigatus, Aspergillus terreus, Aspergillus nidulans, Neurospora crassa, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Magnaporthe grisea, Candida albicans* and *Cryptococcus neoformans* available at NCBI. All the analysis were carried out at an expect value less than 1×10^{-3}.

RESULTS AND DISCUSSION

Two hundred cDNA clones were randomly selected for sequencing to generate 52 novel ESTs, submitted to the EST database (dbEST) at GenBank. Minimum, maximum and average length of sequences was 146, 1287 and 438 respectively.

Assignment of putative function to *A. fumigatus* ESTs: Among the 8 ESTs corresponding to hypothetical proteins of *A. fumigatus*, 2 (TMS25 and TMS45) were assigned putative function on the basis of BLASTX results and 2 other (TMS30 and TMS50) were assigned the function by searching the complete protein sequence against protein family based hidden Markov models (http://blast.jcvi.org/web-hmm/). Interestingly, two of the ESTs (TMS27 and TMS33) did not show homology with any of the predicted CDSs of *A. fumigatus*, however, it showed a perfect alignment with a part of *A. fumigatus* genome. Further experiments need to be done to confirm their expression and role in *A. fumigatus* biology.

Mapping of exon-intron boundries and UTRs: Seven of the ESTs contain partial sequence of 5'UTR whereas three contain partial 3'UTR for different genes (Table1). Sixteen intron-exon boundaries have been mapped for 13 genes (Table1).

Identification of complete CDS for *Tetratricopeptide repeat domain protein* gene: One of the ESTs was comprised of complete CDS for *tetratricopeptide repeat domain protein* gene and submitted the sequence to NCBI database (NCBI Accession no. AAW78029). The complete CDS comprised of 3 exons and 1287 nucleotides codes for a protein of 428 amino acids. The estimated molecular weight and pI (estimated using editseq-DNASTAR software) for the expressed protein are 48.26 kDa and 4.99 respectively. Conserved domain search

Table1: *In silico* analysis of expressed sequence tags of *A. fumigatus*

EST	Gene ID, (Name), [Splice site mapped], {UTR identified}	Homologues in other fungi*	Human homologue
TMS53	Afu1g04070, (Eukaryotic translation initiation factor eIF-5A), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS52	Afu1g14300, (Fasciclin domain family protein), [-], {-}	Afu, An, At, Nc, Cn	+
TMS51	Afu3g06460, (hypothetical protein), [3], {-}	Afu, An, At, Nc, Sp	-
TMS50	Afu8g00780, (putative stage V sporulation protein K), [-], {-}	Afu, At	-
TMS49	Afu4g06160, (branched-chain amino acid aminotransferase, cytosolic), [-], {-}	Afu, An, At, Nc	-
TMS48	Afu4g11800, (alkaline serine protease Alp1), [1], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	-
TMS47	Afu6g04265, (hypothetical protein), [-], {-}	Afu, An, At, Nc	-
TMS46	Afu1g15730, (40S ribosomal protein S22) , [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS45	Afu5g03580, (potential intra-Golgi transport complex subunit 2, COG2), [-], {-}	Afu, An, At, Nc, Sp, Ca, Cn	+
TMS44	Afu7g01930, (GTP-binding protein EsdC) , [-], {-}	Afu, An, At, Nc	+
TMS43	Afu4g10200, (transcription factor RfeF) , [-], {-}	Afu, An, At	+
TMS42	Afu2g14670, (eukaryotic translation initiation factor 3 subunit EifCd), [-], {-}	Afu, An, At, Nc, Sp, Cn	+
TMS41	Afu5g10550, (ATP synthase F1, beta subunit), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS40	Afu2g13530, (translation elongation factor EF-2 subunit), [2], {-}	Afu, An, Nc, Sc, Sp, Ca	+
TMS39	Afu6g04740, (actin Act1), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS38	Afu5g04210, (ubiquinol-cytochrome C reductase complex core protein 2), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS37	Afu4g07200, (hypothetical protein), [1], {5’ UTR }	Afu, An, At, Nc, Ca	-
TMS36	Afu6g03820, (nascent polypeptide-associated complex (NAC) subunit, putative), [1], {-}	Afu, An, At, Nc, Sc, Sp, Cn	+
TMS35	Afu3g11070, (pyruvate decarboxylase PdcA), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	-
TMS34	Afu2g09350, (endo-beta-1,6-glucanase), [-], {-}	Afu, An, At	-
TMS33	No gene homologue; but good match with genomic region of *A. fumigatus* ?	-	-
TMS32	Afu1g07530, (adenylate kinase), [-], {5’ UTR }	Afu, An, At, Nc	-
TMS31	Afu7g05310, (splicing factor u2af large subunit), [1], {-}	Afu, An, At, Nc, Sp, Ca, Cn	+
TMS30	Afu6g13470, (putative Lactamase), [-], {-}	Afu, An, At, Nc, Cn	-
TMS29	Afu2g15290, (DUF636 domain protein), [1], {3’ UTR }	Afu, An, At, Nc	-
TMS28	Afu5g11580, (transcription factor TFIIH subunit Tfb4), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca	+
TMS27	No gene homologue; but good match with genomic region of *A. fumigatus* ?	-	-
TMS26	Afu2g08540, (DNA directed RNA polymerase II 15 kDa subunit), [1], {-}	Afu, At, Nc, Sc, Sp, Ca, Cn	+
TMS25	Afu1g10030 (putative filament-forming protein), [-], {-}	Afu, An	-
TMS24	Afu5g11100, (DUF775 domain protein), [-], {-}	Afu, An, At, Nc, Sc, Sp	+
TMS23	Afu3g10620, (transcription initiation protein), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS22	Afu1g07720, (transcription elongation complex subunit, Cdc68), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS21	Afu6g04740, (actin Act1), [1], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS20	Afu7g04110, (protein kinase C substrate), [-], {-}	Afu, An, At, Nc, Sc, Sp, Cn	+
TMS19	Afu5g02750, (cytochrome c oxidase subunit Va), [-], {5’ UTR }	Afu, An, At	+

EST	Gene ID, (Name), [Splice site mapped], {UTR identified}	Homologues in other fungi*	Human homologue
TMS17	Afu4g11220, (xanthine dehydrogenase HxA), [-], {-}	Afu, An, At, Nc	+
TMS16	Afu3g06300, (Rho GTPase Rac), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS15	Afu5g06130, (succinyl-CoA synthetase alpha subunit), [1], {5' UTR }	Afu, An, At, Nc, Sc, Sp, Ca, Cn	-
TMS14	Afu4g11080, (acetyl-coenzyme A synthetase FacA), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS13	Afu3g05600, (60S ribosomal protein L27a), [-], {-}	Afu, An, At, Nc, Sc, Sp, Cn	+
TMS12	Afu6g12750, (rhomboid family protein), [1], {5' UTR }	Afu, An, At	-
TMS11	Afu7g05530, (DEAD/DEAH box helicase), [-], {-}	Afu, An, At, Nc, Sp	-
TMS10	Afu3g10530, (protein serine/threonine kinase, Ran1), [-], {3' UTR }	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS9	Afu6g10980, (UV-damaged DNA binding protein), [-], {-}	Afu, An, At, Nc, Sp	+
TMS8	Afu1g16280, (mitochondrial F1F0-ATP synthase g subunit), [-], {5' UTR }	Afu, An, At, Nc	-
TMS7	Afu2g14810, (oxidoreductase), [-], {-}	Afu, An, At, Nc, Sc, Sp, Cn	-
TMS6	Afu1g11130, (60S ribosomal protein L6), [1], {-}	Afu, At, Nc, Sc, Sp, Cn	+
TMS5	Afu1g04660, (60S ribosomal protein L15), [1], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS3	Afu6g02470, (fumarate hydratase), [-], {-}	Afu, An, At, Nc, Sc, Sp, Ca, Cn	+
TMS2	Afu1g11220, (GPI anchored protein), [-], {3' UTR }	Afu	+
TMS1	Afu1g07280, (hypothetical protein), [-], {5' UTR }	Afu	-

***Abbreviations.** *Aspergillus fumigatus* (Afu), *Aspergillus nidulans* (An), *Aspergillus terreus* (At), *Neurospora crassa* (Nc), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Candida albicans* (Ca), *Cryptococcus neoformans* (Cn)

was performed using CD-search utility at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). TPR domain was found to span from amino acid 123 to 243. It was identified as a nuclear protein using PSORT 2 program (http://psort.ims.u-tokyo.ac.jp/form2.html). This protein has homologues in other fungi and in human as well. Significant homology (e-value, $<1 \times 10^{-3}$) with approximately 17 human proteins indicates its probable role in triggering autoimmune reactions. Antigenic domain search as performed using protean software (DNASTAR) reveals presence of more of antigenic domains at N-terminal in comparison to those at C-terminal. To examine the presence of IgE epitopes in the protein, we used Algpred software (http://www.imtech.res.in/raghava/algpred/); no IgE binding epitope could be detected in its sequence.

The tetratricopeptide repeat (TPR) is a structural motif present in a wide range of proteins of different organisms ranging from bacteria to humans. It mediates protein-protein interactions and the assembly of multiprotein complexes. Proteins containing TPRs have been shown to be involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport and protein folding (7). In fungi, bimA, a member of the tetratricopeptide repeat family of proteins, has been found to be essential for the completion of mitosis in *A. nidulans*(8); tetratricopeptide repeat (TPR) domain of a Ser/Thr phosphatase gene of *A. oryzae* was necessary for the full activity of the enzyme (9). Reports are available, implicating tetratricopeptide repeat domain in virulence of fungal pathogens; *C. neoformans* with a mutation in the tetratricopeptide repeat-containing gene, *CCN1*, failed to cause systemic infection in mice and regained the virulence when complemented with wild type *CCN1* gene (10). In Candida albicans Tcc1p, a tetratricopeptide domain containing protein, was found to interact with Tup1p to regulate morphological transition and virulence (11). However, the role of tetratricopeptide repeat domain protein in virulence of *A. fumigatus* is yet to be investigated.

Comparative analysis of *A. fumigatus* ESTs with other organisms: *A. nidulans* and *A. terreus* had homologues for 86.5 and 88.5% of ESTs respectively, whereas, *S. cerevisiae* and *S. pombe* had them for 50 and 61.5% of ESTs respectively. This difference is as expected in view of evolutionary divergence between these fungal species. Earlier, we have found this representation to be 89.7% for *A. nidulans* and 61.7% for *S. cerevisiae* using the same cDNA library. Two of the opportunistic fungal pathogens from different class of fungi, *C. albicans* and *C. neoformans* were found to have

homologues for 44.3% and 53.8% of ESTs. For the same cDNA library, representation for 13.23% ESTs has been detected in transcriptome of *C. albicans* (6).

We have found differences in % representation of ESTs (by various fungi) between our two studies and it is also quite different from that reported by Kessler et al (12) , who found sequence similarities of *A. fumigatus* ESTs with *S. cerevisiae*, *S. pombe* and *C. albicans* as 39%, 32% and 38.6% respectively. We believe that this may be due to the fact that number of ESTs in our studies is very small to represent the total coding transcriptome of *A. fumigatus* (9,926 predicted protein-coding genes). Further, conditions used to grow the fungus for RNA isolation might have varied for construction of our cDNA library and that of Kessler et al (12), which, in turn, lead to trapping of different sets of transcripts in the two cDNA libraries.

Identification of antifungal drug targets and virulence factors: Pathogenic proteins should not be having homologues in host proteome to fulfill the criterion of being an anti-pathogenic drug target. On the other hand good homology of pathogenic protein to host protein(s) may lead to autoimmune diseases. Among 19 ESTs having no human homologues,TMS50 code for putative stage V sporulation protein K which along with *A. fumigatus* is only present in *A. terreus*, one of the main causative agents of drug resistant aspergillosis; whereas endo-beta-1,6-glucanase (TMS34) is exclusively present in all three most common causative agents of aspergillosis, *A. fumigatus, A. nidulans* and *A. terreus*. One of the hypothetical proteins (TMS1) is coded exclusively by *A. fumigatus* genome and is absent in other fungal species tested.

Homologues of few of these putative drug targets have already been known as virulence factors in other organisms; adelylate kinase has been implicated in virulence of *Pseudomonas aeruginosa* (13), whereas , succinyl-CoA synthetase has been shown to be important for biofilm formation in bacteria (14). Rhomboid factors are serine proteases (15) and few of the serine proteases have already been known to play a role in virulence of *A. fumigatus* by digesting the host tissue and also by acting as allergen (16). Autoimmune diseases have been known to contribute to the extensive tissue damage observed in allergic bronchopulmonary aspergillosis (ABPA) patients (17). Molecular mimicry between human and pathogenic proteins is one of the proposed mechanisms of autoimmune reactions. Proteins coded by thirty two of ESTs studied here have significant homologies with human proteins, so may be contributing to autoimmune diseases accompanying aspergillosis. TMS52, TMS40 and TMS2 show high degree of homology with human mucin. TMS2, coding for a GPI anchor protein appears to be a potential candidate for triggering autoimmune reactions, as, being a cell wall protein makes it easily accessible by host's immune system and, further, its human homologue being secretory is also easily accessible by the immune system.

Identification of diagnostic markers: Searching for EST homologues in human/fungal sequences provides an account of their utility in PCR based or ELISA based diagnosis of fungal infection(s). TMS50 and TMS34 could be employed for their use in diagnosis of infection by two of the most drug resistant aspergilli sp. (*A. fumigatus* and *A. terreus*) and three of the most common agents of aspergillosis (*A. fumigatus, A. niger* and *A. terreus*) respectively, whereas, TMS33 and TMS1 could be used for diagnosis of infection by *A. fumigatus*. Present study has resulted in rapid and economical identification of 52 ESTs of *A. fumigatus*. The catalogue of ESTs with their probable function and relevance as virulence factors, drug targets or diagnostic markers could serve as a resource for understanding the molecular basis of pathogenicity of *A. fumigatus* and could be useful for development of new therapeutics and diagnostics.

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