Molecular Characteristic and Insilico Analysis of *KatG* Gene in Isoniazid Resistance *Mycobacterium Tuberculosis* Isolate from Sudan

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Received November 05, 2014; Revised December 04, 2014; Accepted December 23, 2014

Abstract The *KatG* gene of *Mycobacterium Tuberculosis* has been associated with isoniazid (INH) drug resistance. While isoniazid (INH) considered as corner stone and main chemotherapy used throughout the world to manage tuberculosis, thus the Progress in apprehension of principle concepts associated with resistance to isoniazid (INH) has allowed molecular tests in addition to bioinformatics tool for the detection of drug-resistant tuberculosis to be developed. In Consecutive isolates (n = 20) of multidrug-resistant *Mycobacterium tuberculosis*, part of the *katG* was sequenced for INH resistance analysis. BLAST analysis of all sequences revealed 100% identity with the available strain “EGY-K361” *Mycobacterium tuberculosis* with Accession No: KC49137.1 except 6 isolates :isolate1, 2, 4, 11, 15, and isolate 20 revealed 99% identity. Thosesix isolates (30%) have detected mutation in Catalase-peroxidase enzyme S315T; three isolate from six 3/6 (50%) of mutant isolates have SNP AGC>ACC substitution while others 3/6 have substitution C>G in position 1280 which may contributed in altering gene expression. The secondary structure of wild and mutant proteins had been done using phyre2 software while the three dimensional structures of them had been done by Chimera software. Stability of mutant protein was increased which detected by i-mutant. Phylogenetic tree of the sequences revealed two distinct phylogroups: mutant isolates and wild isolates phylogroups with controls from different countries retrieved from Gene bank. Serine at position 315 is one of potential drug active sites that proved via SiteEngine soft ware, therefore any substitution will change efficiency of INH.

Keywords: *Mycobacterium tuberculosis*, isoniazid, Sudan, *KatG* gene, S315T

Cite This Article: Gusai H. Abdel Samad, Solima M. A. Sabeel, Walaa A. Abuelgassim, Abeer E. Abdellatif, Wisam M. Osman, Mona A. Haroun, Somaya M. Soliman, Sami. A. B. Salam, Hamid. A. Hamdan, and Mohamed A. Hassan, “Molecular Characteristic and Insilico Analysis of *KatG* Gene in Isoniazid Resistance *Mycobacterium Tuberculosis* Isolate from Sudan.” *American Journal of Microbiological Research*, vol. 2, no. 6 (2014): 227-233. doi: 10.12691/ajmr-2-6-10.

1. Introduction

Tuberculosis (TB) is potential infectious disease [1] caused by *Mycobacterium tuberculosis* (MTB) [2]. It is belong to the order Actinomycetales and family Mycobacteriaceae which are non-motile and non-spore forming acid fast bacilli, characterized by a specialized cell wall that significantly decrease permeability for many compounds [3]. It's contain mycolic acid which break to produce fatty acid methyl esters enable it to resist

disinfectant [4]. Mycobacteria produce degrading enzymes such as β -lactamases and other drug-modifying enzymes [5]. These are among the factors cited to explain the natural resistance of many mycobacterial species to frequently used antibacterial agents [6]. Multidrug-resistant TB (MDR-TB) are resistant to at least rifampin (RIF) and isoniazid (INH) which are the two most important prophylactic drugs against tuberculosis [7]. MDR-TB develops up on treatment of sensitive TB when the course of antibiotics is interrupted either by mismanage or misuse and the levels of drug in the body are insufficient to eradicate all bacteria; This can happen

for a number of reasons: Patients may feel better and halt their antibiotic course, drug supplies may run out or become scarce, health-care providers prescribe wrong treatment, poor quality drug and wrong dose for too short a period of time or patients may forget to take their medication from time to time. MDR-TB is spread from person to person as readily as drug-sensitive TB and in the same manner. INH has a simple chemical structure including a hydrazide group attached to a pyridine ring. Its action is inhibition of mycolic acid biosynthesis [5,8]. It's proposed that INH enters *M. tuberculosis* as a prodrug by passive diffusion and oxidized by mycobacterial catalase-peroxidase, encoded by *katG*, to produce free radicals, which then attack many targets in the cells, therefore mutations in *katG* gene result in failure to generate an active intermediate form of isoniazid [5,8]. The World Health Organization (WHO) estimates about 440,000 new cases of MDR-TB [9], every year it accounts for in excess of 150,000 deaths [10] in response to improper use of the drugs, failure of treatment and transmission of MDR isolates that caused multidrug-resistant tuberculosis (MDR-TB) to become a health catastrophe in developing and developed countries [11,12]. Sudan is a huge poor country with a history of civil war and diverse population. The country has a high frequent of tuberculosis (TB) with an estimated 50,000 incident cases during 2009 [6]. As existing vaccines, drugs, and diagnostics procedures will be insufficient to control and eliminate TB, therefore substantial effort in both basic science and epidemiological studies will be necessary to promote better tools to eradicate TB [13]. Massive researches have demonstrated that INH resistance is most frequently associated with a specific mutation in codon 315 of *M. tuberculosis* catalase peroxidase *katG* gene [14].

We conducted this study to detect *KatG* mutation(s) that responsible for converting MTB to MDR and to predict protein structure to identify possibility of this resist.

2. Material and Method

The study was done during the year 2005 on patients of tuberculosis at Abu-Anja Teaching Hospital Omdurman province. 6736 patients visited the out-patient clinic 4699 (69.7%) of them were males and 2037 (30.2%) females. Only 431 patients were admitted to the hospital, 322 males represented 74.7% while 109 females represented 25.3%.

3. Methodology

3.1. M. Tuberculosis Isolates

Samples of sputum from in-patients suffering from pulmonary tuberculosis were collected, pretreated with 4% NaOH for decontamination and homogenization, neutralization and centrifugation then cultivated on Lowenstein-Jensen media (LJ).

A total of 305 clinical isolated strains of *Mycobacterium tuberculosis* were obtained and identified by conventional biochemical tests (Growth rate, growth at 25°C and 37°C, 68°C labile catalase test, nitrates reduction test, growth on Para-nitrobenzoic acid (PNB) medium, production of pigments and oxygen preference test). As *Mycobacterium* complex collection, transport, pretreatment of samples,

inoculation, incubation, observation, reading and reporting of growth were done according to the WHO instructions. Antimicrobial susceptibility test (proportion method) was performed to detect MDR-TB strains. Their genomes DNA were extracted, the target genes were amplified by PCR, and the hot regions in the *katG* gene were analyzed by automated DNA sequencer.

3.2. PCR Amplification

In PCR forward primer was 22 nucleotide {5'-GCGACGCGTGATCCGCTCATAG-3'} while reverse primer was 16 nucleotide {3'-TCGGCGGTCACACTTT-5'}. Denaturation 30 cycles for 20 seconds, annealing 35 cycles at 55°C and extension 35 cycles at 72°C for 1.5 minutes. PCR products 389 nucleotide were run on agarose gel electrophoresis for confirmation of the results.

3.3. In Silico Analysis

Consecutive isolates (n = 20) of INH resistance *Mycobacterium tuberculosis* from Sudanese patients were sent for sequencing part of the coding region of *katG* for INH resistance analysis. The sequences of 20 MTB isolates achieved were searched for sequence similarity using nucleotide BLAST [15] (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit [16] software. In GeneMark Sversion 4.25 (<http://exon.gatech.edu/genemark/genemarks.cgi>), the gene sequences were translated into amino acid sequence [17]. Sequences similarities were searched with BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi> CMD = Web&PAGE_TYPE=blastDocs) [15] highly similar sequences were achieved from NCBI and subjected to multiple sequence alignment and evolutionary analysis using the BioEdit software which is a mouse-driven, easy-to-use sequence alignment editor and sequence analysis program [16]. phylogeny.fr (<http://www.phylogeny.fr/>) online software were used to draw phylogenetic tree [18] with Clustal Omega to retrieve suitable format [19]. I-mutant version 3 had been used to predict stability of proteins [20]. Expasy PDB sequences that produced significant alignment with the raw sequences were chosen as templates for modeling. PDB code: 1SJ2. The secondary structures of predicted amino acids were carried out by phyre2 [21]. Chimera version 1.8 software used to predict the tertiary model of protein [22]. Predicted model was confirmed with project hope online software data was collected using information from WHAT IF Web services, the Uniprot database and a series of DAS-servers <http://www.cmbi.ru.nl/hope/report/2064?10> [23]. SiteEngine was predicted regions that can potentially function as binding sites to drugs (bioinfo3d.cs.tau.ac.il/SiteEngine) [24] while Active site predication software determine drug binding sites in tertiary structure of protein (<http://www.scfbio-iitd.res.in>) [25].

4. Result

4.1. Antimicrobial Sensitivity

Conventional indirect susceptibility testing against rifampicin and isoniazid (proportion method) was

performed for the previously obtained isolates and the result was as follows:-

238 isolates were sensitive to both rifampicin and isoniazid (92.5%), 20 isolates were resistant to both rifampicin and isoniazid (6.5%), 02 isolates were resistant to isoniazid only (0.66 %), 01 isolate was resistant to rifampicin only (0.33 %), Multidrug resistance was found to be 6.5%.

The sequences of the PCR products were of the same gene (catalase peroxidase from twenty resistant isolates). The consensus sequence codes for amino acids 271-399 of catalase peroxidase. Only one difference was found (G245C) within all the sequences that leads to a S315T mutation in the wild-type protein. The rest of the sequences seem to be identical.

BLAST:

Table 1. show the Basic local Alignment of the sequences obtained from sequencing. Identities were searched against DNA Mycobacterium tuberculosis EGY-K361" control strain [Accession NO. KC491371.1] and catalase-peroxidase of Mycobacterium tuberculosis [Accession NO. AGJ83814.1] for translated sequences

Strain NO	DNA Residue	Identity DNA	Amino acid residue	Identity
Isolate-1	310	99%	102	98%
Isolate-2	314	99%	104	99%
Isolate-3	311	100%	103	100%
Isolate-4	310	99%	102	98%
Isolate-5	310	100%	102	99%
Isolate-6	300	100%	99	100%
Isolate-7	311	100%	103	100%
Isolate-8	310	100%	103	100%
Isolate-9	301	100%	100	100%
Isolate-10	318	100%	106	100%
Isolate-11	310	99%	103	99%
Isolate-12	308	100%	102	100%
Isolate-13	317	100%	105	100%
Isolate-14	339	100%	113	99%
Isolate-15	339	99%	113	98%
Isolate-16	341	100%	113	99%
Isolate-17	322	100%	107	100%
Isolate-18	338	100%	113	99%
Isolate-19	338	100%	113	99%
Isolate-20	340	99%	113	98%

4.2. In Silicomodellingof (katG) Coding Protein

GeneMark S

The nucleotide sequences obtained was translated into aminoacid sequences using GeneMark S version4.25 which is a family of gene prediction programs developed at Georgia Institute of Technology, Atlanta, Georgia, USA. It is software used to translate DNA sequence to amino acid <http://exon.gatech.edu/genemark/genemarks.cgi> and residues of amino acids determined in Table 1.

BLASTp

The aminoacids of the protein sequences were compared against the protein databank database using BLASTp. The 20 mycobacterium tuberculosis isolates were compared against the PDB protein database. The sequences produced significant alignment with catalase-peroxidase of Mycobacterium tuberculosis [Accession NO. AGJ83814.1]determined in Table 1.

BioEdit:

The multiple sequence alignment of the isolates with similar nucleotide sequences obtained from BLAST was carried out to find the homology and evolutionary relation between the sequences. The numbers of conserved regions were detected by BioEdit version 7.0.9.0. The nucleotide sequence alignment reveals 12 conserved regions. 3/6 isolates 1, 4 and 11 showed transversionfrom guanine Gnucleotide into cytosine C(AGC>ACC). Translated sequences aligned with similar protein databank sequences obtained from BLASTp Finland ID: AAA85177.1, IndiaID: AGR27787.1, South Africa ID: JX303272.1, Pakistan ID: AEV59559.1, Poland ID: gb|AHA61381.1, France ID: AGF25369.1, Sweden KCR29412.1, South KoreaKCF64587.1, China AAY54554.1 and Egypt ID: AGJ83815.1 used as controls. Six isolates 1,2,4,11,15 and 20 have mutation that convert serine(SER) into amino acid Threonine(THR) at codon 315 {S315T}.Amino acid sequence alignment revealed two conserved regions among whole sequences.

Figure 1. multiple sequence alignment of 20 MTB DNA sequence and different MTB strains

Figure 2. multiple sequence alignment of 20 MTB translated sequence and different MTB strains

Clustal Omega

The multiple sequence alignment of the isolates was done to achieve the homology and evolutionary relation between the sequences then the Newick Format was transfer to phylogeny.fr software.

Phylogeny.fr

Software used to draw phylogenetic tree which revealed the evolutionary relation between the sequences. The

phylogenetic analysis of the sequences revealed two distinct phylogroups which include the MTB mutant isolates 1, 2, 4, 11, 15, 20 with Thailand and Pakistan isolates in a single group and all other MTB wild isolates 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, 17, 18 and 19 with available Gene bank Finland, China, India, Egypt and South Africa isolates isolate in another phylogroups.

Figure 3. Phylogenetic analysis tree of 20 MTB isolates and different controls retrieved from database

phyre2

The secondary structure of the protein sequences was predicted from the translated sequence using phyre2 online software.

Figure 4. predicted secondary structure of the wild type protein sequences

Figure 5. predictedsecondary structure of the mutant protein sequences

Figure 4 illustrated the protein secondary structure of wild MTB isolate contains 15% alpha helix and 13% beta strands. Figure 5 illustrated the protein secondary structure of mutant MTB isolate contain 14% alpha helix and 11% beta strands.

4.3. Tertiary Structure Predicting of Sequences

Chimera version 1.8 was used for the predicting of 3D structure of the raw amino acid sequence.

Figure 6. left: show wild type of predicted amino acid serine SER at position 315 of mycobacterium tuberculosis katG gene (small) Right: show mutant type of predicted amino acid threonine THR at position 315 (large)

SiteEngine software

The SiteEngine software it is functional Sites Structural Search Engine that detect 1500 binding sites select part of them randomly from whole result shown in Table 2 reveal that serine at position 315 is one of potential active sites, therefore any substitution will change efficiency of drug.

Table 2: Detect part of drug binding sites using SiteEngine software serine at position 315 one of drug binding sites appear in red color and denoted by red color arrow. A.A - Amino acid DON - Hydrogen bond donor, ACC - Hydrogen bond acceptor, DAC: Hydrogen bond donor and acceptor, ALI - Aliphatic Hydrophobic property, PII - Aromatic property, S: site chain of amino acid, b: backbone, Dis: distance in space measured between the matched features, * star sign indicate conserve A.A region.

Table 2.

	Chain ID	A.A	Property	Source	Distance	Conserve A.A
	A.91	Trp	DON	s	6.9e-12	
	A.94	Asp	DON	b	7.4e-12	
	A.97	His	ACC	b	7.7e-12	
	A.100	Pro	ACC	b	6.3e-12	
	A.101	Leu	DON	b	6.6e-12	
	A.103	Ile	ALI	s	7e-12	*
	A.270	His	ACC	b	5e-12	*
	A.275	Thr	ALI	s	3.4e-12	
	A.276	His	PII	s	5e-12	*
	A.314	Thr	ACC	b	3.3e-12	
→	A.315	Ser	ACC	b	2.5e-12	
	A.317	Ile	ALI	s	2.6e-12	
	A.380	Thr	DAC	s	4.4e-12	

Active site predication software

The drugs binding sites were visually confirmed in 3D structure using Active site predication software.

Figure 7. Arrow denote to drug binding site in 3D structure of predicted protein by Active Site prediction software

5. Discussion

Tremendous efforts have been made in drug of tuberculosis, however much remains to be learned about the molecular basis of drug resistance in Mycobacterium tuberculosis. In our study 6 isolates (30%) out of 20 mycobacterium tuberculosis were found to be INH resistant due to S315T mutation in catalase-peroxidase encoded by KatG gene. This result is similar to that reported in Barcelona which alter in the katG gene were detected in 55% of the 61 Mycobacterium tuberculosis isoniazid-resistant isolates. Mutation in codon 315 was the

most prevalent (32%). Strains showed a high level of resistance, and most maintained a substantial catalase-peroxidase activity [26].

Also another study in Iran had 3 isolates out of 10 isolates showed mutation in codon 315 by sequencing these were accounted for 30% of total katG mutation determined by Multiplex allele-specific PCR [27].

Additional study in India reveal that Among seventy-four clinical isolates of *M.tuberculosis* three MDR-TB isolates showed transversion of codon 315 AGC-ACC (Ser-Thr) in the sequenced katG region for INH resistance. The prevalence of mutations at codon 315 differ according to geographical regions [28].

In contrast further study in China, (82%) of isolates were found to have the same mutation, in which 32 isolates were have AGC>ACC transversion and the rest nine isolates were have AGC>AAC transition [29], while in our study, 3 out of 6 S315T mutant isolates showed AGC>ACC and others 3/6 have substitution C>G in position 1280 which may contributed in altering gene expression.

The Structure of mutant protein tend to be in another secondary structure, therefore the local conformation will be slightly destabilized. The wild-type and mutant amino acids vary in size; the mutant protein is larger than the wild-type. The wild type residue forms a hydrogen bond with the Isoleucine on position 317. The size difference between wild-type and mutant residue makes that the new residue is not in the correct position to make the same hydrogen bond as the original wild-type residue did. According to position analysis the mutant residue is located on the surface of the protein, therefore can disturb interactions with other molecules or other parts of the protein. It is located near a highly conserved position.

6. Conclusion

Obviously from the results that the dispersal of insilico tools for application in biomedical research have great impact and has huge effect on the ability to understand genetic differences which may contribute with drug resistances also S315T may be used as rapid screening markers for early diagnosis of the disease and the fast identification method for recognizing isoniazid resistant strains which is essential for efficient treatment and control of the MDR strains. Further investigation of these isolates is needed to identify whether the addition of one or more targets in other genes would increase the sensitivity of the assays

Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

This study was supported by the University of Bahri (Sudan), we thank all the families, patients and control individuals for their cooperation in this study

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