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Molecular marker analysis kala-azar: An *insilico* approach

Chikoo Cherry Abraham Cherian, J. Jannet Vennila, S.Ganesh,

Department of Bioinformatics, Karunya University, Coimbatore, Tamil Nadu, India

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ABSTRACT

Kala-azar caused by Leishmania donovani species complex is endemic in 88 countries. Kala-azar is transmitted by sandflies (Phlebotomus species). This project aimed to identify molecular markers for Kala-azar (gene and protein) and to develop a biodiagnostic tool for leishmaniasis by insilico method based on molecular markers. With the help of bioinformatics tools, databases and approaches, the studies on pathogenesis of disease involving both Leishmania donovani and homo sapiens(human) were carried out. For identifying the suitable gene marker, the microarray data analysis was referred and a list of Leishmania genes were displayed. Of that, CFAS(cyclopropane fatty acyl phospholipid synthase) was taken into consideration due to its absence in the human genome and high gene expression rate. Using CFAS (cyclopropane fatty acyl phospholipid synthase) protein as target, and Salicylic acid, a phytochemical from Aloe vera as ligand was docked, but the result showed less docking score than a natural inhibitor Sinefungin. This shows that CFAS (cyclopropane fatty acyl phospholipid synthase) may not be used as a drug target but, it can be used as a biomarker for Kala-azar because high level of expression was noted in microarray expression studies. Therefore, a suitable method to use CFAS as a marker protein to diagnose Kala-azar was proposed.

Keywords : Kala-azar, *Leishmania donovani*, Visceral Leishmaniasis, CFAS, Molecular markers.

INTRODUCTION

Kala-azar or Visceral leishmaniasis is caused by the protozoa *Leishmania donovani*. It is transmitted by the vector sandfly. Leishmania is a vector-borne pathogenic parasite found in 88 countries worldwide and is the causative agent of the disease Kala-azar. [2]. The different Leishmania species infect macrophages and dendritic cells of the host immune system and affects the haemopoietic organs. Field diagnosis is difficult in the absence of simpler and less invasive tests with good sensitivity and specificity [7]. The existing parasitological tests from marrow aspirates and spleenic aspirates require are invasive and unsuitable for all patients. The field of diagnosis is improving and newer approaches offer great promise in making the diagnosis specific and sensitive [3].

Visceral leishmaniasis (VL), also known as kala-azar, black fever, and Dumdum fever [4]. Is the most severe form of leishmaniasis. Leishmaniasis is a disease caused by protozoan parasites of the *Leishmania* genus. After malaria, this disease is the second-largest parasitic killer in the world. It is responsible for an estimated 500,000 cases each year worldwide [22]. This parasite migrates to the internal organs such as liver, spleen (hence '*visceral*'), bone marrow and causes fever, weight loss, mucosal ulcers, fatigue, anemia and substantial swelling of the liver and spleen, if untreated result in the death of the host [6]. Leishmaniasis is caused by protozoa of the genus *Leishmania* and transmitted to humans by sandflies. Leishmanial infection has a wide spectrum of manifestations, including asymptomatic infection, cutaneous leishmaniasis, mucous leishmaniasis and visceral leishmaniasis (VL; also known as "kala-azar") [5]. Acute cases of Kala- azar cause the enlargement of the visceral organs like the spleen and the liver. The disease causes its infection and ultimately results in the death of the patient [12]. The identification of a CFAS gene in Leishmania raises the exciting possibility that some virulence factors are conserved between bacterial and eukaryotic intracellular pathogens. The interesting part of this gene is that it is completely absent from the human genome [8]. The use of traditional herbs has proved to be highly effective in fighting the disease Kala-azar. *Aloe vera* is one such example as it inhibits the promastigotes and amastigotes that is responsible for the proliferation of the disease. The different phytochemicals present in *Aloe vera* namely aloin, glucumannons and salicylic acid prove to be highly effective in treating the disease [11].

Identified molecular markers for Kala-azar (gene and protein). Found plant based phytochemicals that can treat Kala-azar and Developed a biodiagnostic tool for leishmaniasis by *insilico* method based on molecular markers.

MATERIALS AND METHODS

The study was started in December 2010.

Microarray data analysis

The microarray database is usually used to describe containing microarray gene expression data [10]. The organism *leishmania donovani* was selected in the Select Organism space. In the Category space, Gene Overexpression was selected. Then the display data was clicked. The list of the *leishmania* genes that were expressed was studied. One of the novel gene which was found to be highly expressive in *Leishmania donovani* was CFAS (cyclopropane fatty acyl phospholipid synthase).

Identification of novel gene (marker)

The differential expression pattern which is unique to *L. donovani* was mainly focussed by the GEO (Gene Expression Omnibus) Profile Database. The GEO Profile Database was accessed from the internet at the website http://www.ncbi.nlm.nih.gov. The gene CFAS was entered in the lower Search space region. The expression rate of the gene was observed.

Structural studies of protein

The structural studies of protein encoded by the gene by *insilico* method was mainly focused by PDBSUM, J MOL Viewer and FAST A format and Q SITE FINDER.

Docking the protein by natural active compounds (phytochemicals)
AutoDock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure [13]. CFAS was taken as the target template to which the phytochemicals from aloe vera which act as the ligands were docked and was compared to its natural inhibitor sinefungin.

Kala-azar diagnosing
To propose a suitable method for the diagnosis of Kala-azar, a Kala-azar diagnosing kit can be prepared using CFAS. Blood sample containing the leishmania is one crucial requirement for this kit. A fluorescent microscope can be used to determine the presence of the CFAS gene.

RESULTS

Identification of novel gene (marker)
All the *leishmania* proteins listed in Table 1 were also present in humans except Cyclopropane fatty acyl phoshpholipid synthase (CFAS). Therefore, CFAS was given special emphasis in identification of molecular markers and also drug target in Kala-azar. The expression details of the gene were derived with the help of GEO Microarray database. The expression rate of the gene is in the range of 75-80 % which is an optimum value for its selection.

Structural studies of CFAS protein
The 3D structure of CFAS protein (1kp9) was retrieved from PDB in J MOL viewer which is represented in Figure 1.

Figure 1: Structure of CFAS protein

Sequence analysis of CFAS protein
The FAST A format was used to visualize the sequence of the protein 1kp9. This enabled the user to find the active regions of the protein and it is shown in Figure 2.

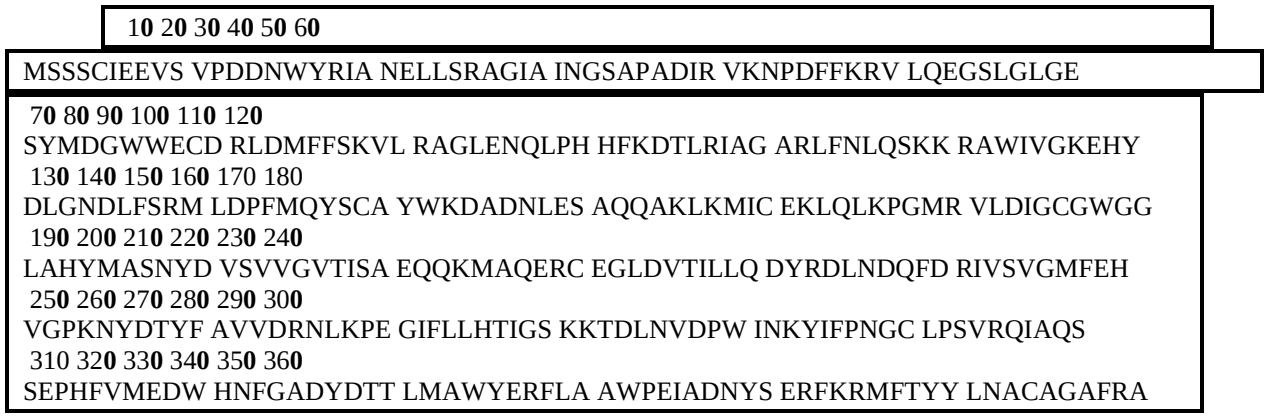

Figure 2: Sequence analysis of CFAS protein

Table 1: Residues in the active site of CFAS protein

Position number	Atom	Residue	Residue number
1211	N	GLY A	171
1212	CA	GLY A	171
1213	C	GLY A	171
1214	O	GLY A	171
1215	N	LEU A	172
1216	CA	LEU A	172
1217	C	LEU A	172
1218	O	LEU A	172
1219	CB	LEU A	172
1220	CG	LEU A	172
1222	CD2	LEU A	172
1223	N	HIS A	173
1224	CA	HIS A	173
1225	C	HIS A	173
1226	O	HIS A	173
1227	CB	HIS A	173
1228	CG	HIS A	173
1229	ND1	HIS A	173
1230	CD2	HIS A	173
1231	CE1	HIS A	173
1232	NE2	HIS A	173
1233	N	PRO A	174
1234	CA	PRO A	174

Non bonded interactions in Sinefungin

Six non bonded interactions between the active site of CFAS protein and the inhibitor sinefungin were observed. The major residues that were involved in the non bonded interactions were Glycine 72, Tryptophan 75, Aspartine 20, Leucine 18 and Glutamine 140.

Non bonded interactions in Salicylic acid

Two non bonded interactions between the active site of CFAS protein and salicylic acid were found. Ileucine 136 and Tyrosine 33 was the major residue that was involved in the non bonded interactions.

Propose a suitable method to use this marker protein in diagnosis of Kala-azar

Kala-azar diagnosing Kit (KDK) can be prepared with CFAS protein which act as an antigen. One drop of Blood sample (serum) is added. If it is positive for Kala-azar, the serum would

contain a primary antibody against CFAS. The CFAS antibody then reacts with the CFAS protein. The antibody which acts as a secondary antibody fluorescently labelled, is added to the above mixture. The secondary antibody then binds to the primary antibody which shows fluorescence after binding. The fluorescence can be detected by fluorescent microscope.

DISCUSSION

Molecular markers have acted as valuable tools and have found their own position in various scientific fields like characterization of genetic variability, genome fingerprinting,genome mapping, gene localization, analysis of genome evolution, population genetics [18]. The identification of a CFAS gene in Leishmania raises the exciting possibility that some virulence factors are conserved between bacterial and eukaryotic intracellular pathogens. The interesting part of this gene is that it is completely absent from the human genome [8]. Due to the absence of the CFAS gene in the human genome, this gene can be used as a potential marker to detect Kala-azar which is unique and much more effective than other leishmanial genes because of its presence in the human genome. Microarray technologies provide new approaches to study the pattern of gene expression profile. It has the potential to improve our understanding of pathogenicity, mechanism of drug resistance and virulence factors by identifying up/down regulated gene and characterizing the respective gene expression. Microarray technology is one approach that can be used to find the genes responsible for the disease Kala-azar. The approach was to compare the gene expression of both forms of the parasite and characterize transcripts developmentally expressed in amastigotes. Microarray will be helpful for defining effective targets. The morphological and physiological changes of the *Leishmania* parasite which occur following its transfer from the sand fly to a mammalian host suggest a rapid modulation of the expression of numerous genes. To identify molecular events associated only with the amastigote stage of *Leishmania* parasites, expression profiling has been used. The approach was to compare the gene expression of both forms of the parasite and characterize transcripts developmentally expressed in amastigotes. [1]. Using GEO Profile Database, the gene expression of CFAS was studied and the gene expression rate was 75-80 percent which is quite suitable for its selection as a gene marker. Sinefungin, a natural nucleoside isolated from cultures of *Streptomyces incarnatus* and *S. griseolus*, is structurally related to S-adenosylhomocysteine and S-adenosylmethionine. Sinefungin has been shown to inhibit the CFAS active site. This compound has been reported to display antiparasitic activity against leishmanial species. Sinefungin was significantly suppressive against both *L. donovani* infections [9]. This natural inhibitor was taken into consideration and its docking sites with the gene CFAS was compared with the phytochemicals taken from the plant Aloe vera which was docked into the CFAS gene. The use of traditional herbs has proved to be highly effective in fighting the disease Kala-azar. *Aloe vera* is one such example as it inhibits the promastigotes and amastigotes that is responsible for the proliferation of the disease [19]. *Aloe vera* L. is a versatile medicinal plant with many cosmetic properties. It has been shown that the plant have antiseptic, anti-tumoral, anti-inflammatory, anti-oxidant and immuno-stimulant activities. Compounds from Aloe are also reported to show anti-bacterial , anti-viral , anti-fungal and cell growth stimulatory activity . The juice of plant is considered useful in gastrointestinal diseases and promotes healing . This plant is an important medicinal plant [17]. Based on the above study, the different phytochemicals from aloe vera are isolated which included aloin and salicylic acid. Aloin had to be rejected because of its large molecular structure and salicylic acid had to be rejected because of only minimal number of docking sites with the CFAS gene. But it has been proved that other phytochemicals from aloe vera can be used to dock with the active site of the CFAS gene and hence it can be successfully used as a drug target and a molecular target. The commercial antibiotics involve AmBisome which is commercially recognized for the treatment of Kala- azar. The studies on the chemical

structure of AmBisome shows that it can be used as an effective drug for the treatment of Kala-azar. AmBisome is a true single bilayer liposomal drug delivery system, consisting of unilamellar bilayer liposomes with amphotericin B intercalated within the membrane. Amphotericin B, the active ingredient of Am**B**isome, acts by binding to the sterol component of a cell membrane, leading to alterations in cell permeability and cell death [14]. This drug due to its effectivity in treating the disease was docked with the CFAS protein, but because of its large molecular structure, this analysis had to be rejected. Salicylic acid a phytochemical derived from aloe vera is a monohydrobenzoic acid a type of phenolic acid and a beta hydroxyl acid [16]. Salicylic acid shows interactions with the CFAS protein which binds to its active site on docking .Two non bonded interactions between the active site of CFAS protein and salicylic acid were found. Ileucine 136 and Tyrosine 33 was the major residue that was involved in the non bonded interactions. But compared to the natural inhibitor sinefungin it showed comparitively less number of interactions. Therefore it had to be rejected. But this led to the study that other phytochemicals isolated from aloe vera can be docked into the CFAS protein to inhibit its active site. Aloin extracted from aloe vera is a mixture of two diastereomers, termed aloin A (also called barbaloin) and aloin B (or isobarbaloin), which have similar chemical properties [15]. Aloin is an anthraquinone glycoside, where the skeleton has been modified by the addition of a sugar molecule [18]. Aloin could not be docked with the CFAS template because of its large molecular structure. Therefore, this phytochemical had to be rejected. But this too led to the study that other phytochemicals from Aloe vera can be docked into the CFAS protein to inhibit its active site and use it as a drug target.

CONCLUSION

Kala-azar also known as Visceral leishmaniasis is endemic affecting 12 million people and is transmitted by sand fly of the Phlebotomus species. For the identification of this disease a marker protein was identified namely CFAS (cyclopropane fatty acyl phospholipid synthase). The CFAS protein is located in the cytoplasm as well as in the plasma membrane of the cell and its biological processes involves lipid biosynthetic process, methylation S-adenosylmethionine metabolic process and mycolic biosynthetic process. The CFAS protein is an enzyme that has the substrates S-adenosyl-L-methionine and phospholipid olefinic fatty acid, whereas its two products are S-adenosylhomocysteine and phospholipid cyclopropane fatty acid. Using these substrates, the Kala-azar diagnosing kit can be prepared. Kala-azar diagnosing Kit (KDK) can be prepared with CFAS protein which acta as an antigen. One drop of Blood sample(serum) is added. If it is positive for Kala-azar, the serum would contain a primary antibody against CFAS. The CFAS antibody then reacts with the CFAS protein. The anti antibody which acts as a secondary antibody fluorescently labelled, is added to the above mixture. The secondary antibody then binds to the primary antibody which shows fluorescence after binding. The fluorescence can be detected by fluorescent microscope.

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