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Evolution of a mammalian specific post-translationally regulated moonlighting function in Glyceraldehyde-3-phosphate dehydrogenase

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Abstract : Moonlighting proteins are proteins that exhibit multifunctionality. It is accomplished by the recruitment of already present domains to new functions during evolution. Some functions may be due to the effect of Post Translational modifications (PTM). Glyceraldehyde -3 phosphate dehydrogenase (GAPDH) is a highly conserved housekeeping enzyme that has multiple moonlighting functions. Along with its primary function of glycolysis, it also performs secondary moonlighting functions such as apoptosis, negative regulation of translation, and killing of cells of other organisms.

We were interested in finding how the moonlighting functions due to PTM in GAPDH evolved among different organisms.

Multiple sequence alignment (MSA) was done on GAPDH sequences retrieved from the Moonprot database, and PTMs were plotted on it. We also constructed a phylogenetic tree using the ML method. Our results suggest some motif [**IL]-x-C-x-x-[DE]** and associated PTM is present only in mammals, indicating that apoptotic moonlighting function may have evolved only in mammals, at a later stage.

Keywords: Moonlighting proteins, Glyceraldehyde - 3 phosphate dehydrogenase (GAPDH), post-translational modification, Phylogenetic tree

1.INTRODUCTION

Moonlighting protein is a protein which can perform more than one function [12]. Moonlighting proteins originally may have had only a single function, eventually through evolution, they may have acquired additional functions [13]. Many moonlighting proteins are enzymes. Though in these enzymes the common primary function is enzymatic catalysis, these enzymes have acquired secondary non-enzymatic roles [13]). Some examples of functions of moonlighting proteins secondary to catalysis include signal transduction, transcriptional regulation, apoptosis, motility, and structural [13].

Moonlighting proteins are found distributed widely among many organisms. Though different proteins can be produced by a single gene by alternative RNA splicing, DNA rearrangement, or post-translational processing, moonlighting protein exhibits different functions due to different domains in a single protein. It can also be called a multi-functioning protein. These multifunctions are because of multiple domains which carry out different functions independently of each other. Gene Sharing is said to be a single gene responsible for different functions [and moonlighting proteins exhibit this gene sharing [14].

1.1 Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) is also known as Peptidyl Cysteine S nitrosylase (GAPDH), which is an enzyme that has multiple moonlighting functions. These multi functions are the result of post translational modifications (PTMs) at various locations in the protein [15]. The enzyme has both their enzymatic and different non enzymatic functions in various organisms. The primary function of GAPDH is Glycolysis, glycolysis is carried out in the cytoplasm of the cell. The secondary moonlighting functions are carried out after the enzyme is post translationally modified [15].

1.2 Post-translational modification

Protein undergo enzymatic modification to produce protein which further undergoes PTMs to produce mature protein. The multiple functions seen in the GAPDH moonlighting proteins is due to post translational modifications [15]. Post-

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translational modification (PTM) refers to the modification brought to any amino acid on the protein which may be by acetylation, phosphorylation, nitrosylation. Posttranslational modifications (PTMs) are covalent processing events that change the properties of a protein; they may activate the protein and help in specific functions. In GAPDH Biological behaviour such as protein-protein interaction, the cellular process is regulated by post-transcriptional modification. There are 3 types of ptms they are phosphorylation, O-linked glycosylation and acetylation. [21]

1.3. Binding partners

The multiple functions of PTMs and their regulations are formed to the motif and their corresponding binding partners they bind to PTMs and bring about functions such as nuclear localisation/ Functions are related to that particular motif [20]. Proteins bind to each other through a combination of hydrophobic bonding

1.4. Moonprot database

Information about the structure and function of moonlighting protein is scattered in many publications. In order to collect all information of moonlighting protein manually curated, searchable, the internet-based database is created that is moonprot database. This database provides information about more than 200 proteins that have been experimentally verified. It consists of organised information and gives a complete view of the current moonlighting protein.
Hence we further decided to proceed along with the topic then we found the reason for their multiple functions were Post Translational modification and we found about the motif and then we took a set of organisms listed in the moonprot then we did MSA using muscle tool then we found there are many conserved semi-conserved sequences then we plotted in GAPDH then we plotted ptms on MSA and found that all organisms taken for MSA as this PTMs conserved but due to lack of motif in them only in higher organisms this PTMs and motif bring about Functions such as Apoptosis. further, we did phylogenetic trees and mapped motifs and determined their evolution.

2. METHODS

2.2. Selection of the moonlighting protein and function

GAPDH enzyme was selected for studying moonlighting functions. Search for Moonlighting function on which maximum literature was available was done in order to find and select the function of moonlighting protein.

2.2. Database

The Moonprot database (http://www.moonlightingproteins.org/) was used to get further information regarding GAPDH enzymes' primary and secondary functions of the protein. The Moonprot database contains reported information about the GAPDH in 24 organisms.

2.3. Retrieval of the protein sequences in Fasta format

Accession numbers of the sequences as reported in the Moonprot database were obtained and fasta sequences were retrieved from Uniprot.

2.4. Multiple sequence alignment: The fasta sequences obtained were entered in an online MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/) and Multiple Sequence Alignment (MSA). The resulting MSA was analysed for conserved(motifs), semi-conserved regions for the post-translational modification sites PTMs) [1]. Then the function of each of these PTM's was obtained from earlier reports [2][3][4]. PTMs were plotted on the MSA obtained.

2.5. Phylogeny

For phylogenetic analysis, the "https://www.phylogeny.fr/" tool was used to create a phylogenetic tree. Then plotting PTMs on a phylogenetic tree was done. Phylogenetic analysis was done using the "one-click" option. Phylogeny.fr aligned the sequences using MUSCLE, curated the MSA using G blocks and built the phylogeny using phyML. The tree was rendered using tree Dyn. All these options are set as default in the ‘one-click” mode.
Post-transcriptional modifications were plotted through a diagrammatic representation showing evolutionary pathways and connections among the organisms.
The Phylogeny.fr platform was used.

3. RESULTS:

3.2. Muscle:

Fasta sequences retrieved from the MoonProt database were aligned using the MUSCLE program to see the conservation of regions. GAPDH showed a high degree of conservation across very distantly related organisms in the tree of life. From

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the above obtained MSA result we have 60 conserved sequences **(*)**, 28 semi-conserved sequences **(.),** 39 conserved Mutated sequences **(:)** and the remaining are mutated sequences **()**.

3.2. MOTIFS:

Motifs are small amino acid sequences that participate in a specific function. We analysed motifs involved in nuclear localisation and other cellular functions. [6] MSA revealed a motif in GAPDH - **[IL]-x-C-x-x-[DE].** [7] In GAPDH the motif consists of 6 amino acids **LTCRLE (Fig-3).** This motif is responsible for signal transduction and cell function like apoptosis glycolysis, etc, inducing transglycosylase activity, shuttling of nitric oxide from nitric oxide synthase to target protein.

Fig 3- Fasta sequences retrieved from the MoonProt database were aligned using the MUSCLE program. From this alignment, we found that PTM is present in 7 organisms which are animals. Six are mammals (Human, Bovin, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster) and one is parasite Haemonochus contortus (commonly known as barber's pole worm).

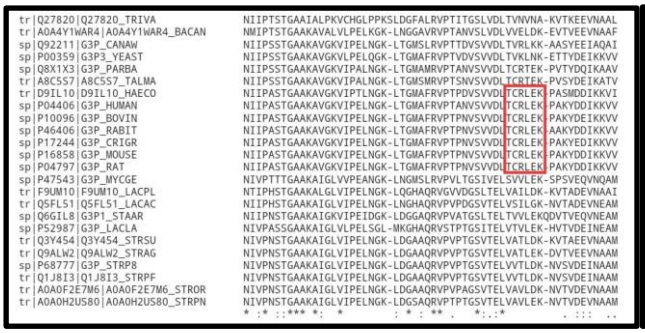

Fig : 3

Fig:4

3.3. Post Translationally modified amino acid.

In order to attribute moonlighting functions to the retrieved sequences, we checked the status of conservation of the amino acid residues that are post-translationally modified. We assumed that the conversation of PTM residue in a set of sequences meant that they performed that particular moonlighting function.

1. LYSINE -117,227,251.

Under apoptotic stress, Nitric oxide is released into the cell. The motif involved is [**IL]-x-C-x-x-[DE].** Which further induces the binding of acetyltransferase P300/CBP associated factor (PCAF). Lysine 117, 227, 251 are found to undergo acetylation [2]. Instead of a direct mechanism in which structures are altered and functions are activated, a series of complex events undergo acetylation [2]. Upon this post-translational modification, GAPDH is transferred to the nucleus from the cytoplasm. [2]

All the three lysines at these positions have to be together modified to achieve this transfer. And in absence of one PTM, this function may be affected [2][3].

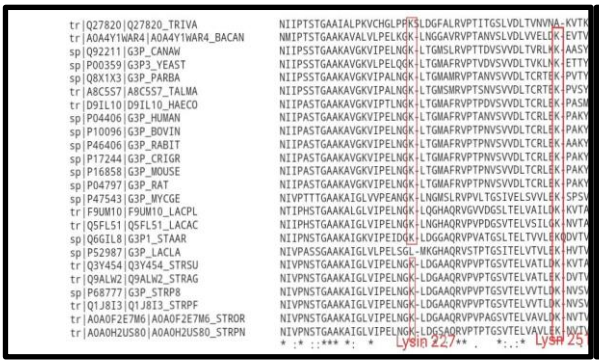

Fig: 5

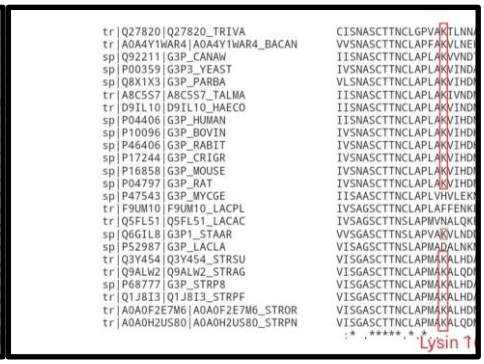

Fig: 6

At 117 positions Lysine is conserved in all organisms but at 227 one organism shows this lysine substituted with Leucine. However, these ptms are present in two bacteria one is (Lactococcus lactis LACLA) the rest of them have conserved in all organisms and at 251 position but at one position (Trichomonas vaginalis) showed absence of this PTM. Even Though

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these PTM amino acids are conserved in bacteria and fungi to initiate the post-translation we required Nitric oxide (NO) which is translocated from the motif **[IL]-x-C-x-x-[DE]** but it's only present in seven organisms. So we deduce that this particular moonlighting function may be absent in bacteria and fungi but only seen in mammals given in the list. (such as Human, Bovin, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster) and parasite Haemonochus contortus (commonly known as barber's pole worm).

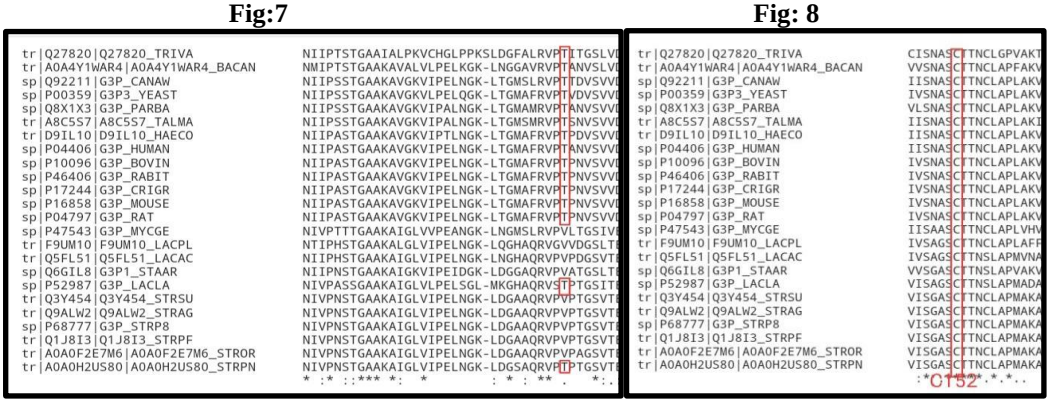
LYSINE - 160

LYSINE 160 is involved in Apoptosis. The apoptotic stress activates nitric oxide synthase which produces nitric oxide which binds to the Saih 1 then bound complex of Saih 1 and GApdh will bring about Apoptosis.

But Nuclear GAPDH is acetylated at lys 160 by acetyltransferase p300/CREB binding partner (CBP) from direct protein interactions which stimulate the acetylation and catalytic activity of the p300/CBP, GAPDH from the cytoplasm is translocated from the cytoplasm to the nucleus, Further other targets of p300/CBP are targeted and brings about cell death The dominant-negative mutated GApdh with the substitution of apoptotic genes helps in decreasing the number of cell death[5][8][3] Lysine 160 PTM is absent in organism **MYCGE(Mycoplasma genitalium) LACPL(Lactobacillus plantarum) LACAC(Lactobacillus acidophilus)** (Fig-6) **, LACLA(Lactococcus lactis),** but because the motif is absent in bacteria and fungi and therefore we predict that these functions are not seen in them. Only in the organisms Human, Bovine, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster) and parasite Haemonochus contortus (commonly known as barber's pole worm) contains [**IL]-x-C-x-x-[DE] motif** is present hence we attribute a putative moonlighting function of apoptosis in them.

3.4. Threonine 237

Fig:7 **Fig: 8**



Akt2 phosphorylated T -237 of GAPDH and decreased its nuclear translocation which is essential for the GAPDH associated Apoptosis. Akt2 is a protein kinase that helps in the regulation of cell growth and survival [4]. The functions of T-237 are inhibition of translocation and pro-apoptotic function [3]. This suggests that Akt2 induced phosphorylation GAPDH plays an important role in blocking Apoptosis. MSA of the sequences shows that nine organisms don't have this ptm and they are all bacteria (Fig-7).

T-237 shows a high degree of variation as can be seen in the MSA. fungi and animals but this function is functional only in Human, Bovin, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster).

3.5. CYSTEINE 152

GAPDH undergoes S- nitrosylation which is brought by the Nitric oxide (NO) which further binds to the SiAH 1 and then translocates the gapdh into the nuclease. This further induces cell death. NO also induces S-nitrosylation cysteine 152 which helps in nuclear translocation and pro-apoptotic function. This is a common ptm located in all 24 moonlighting exhibiting organisms. S-sulfuration of the active site cysteine results in ~7-fold increase in the activity of the enzyme (GAPDH) but they are not functional in bacteria and fungi because NO are transferred because the motif responsible for this is present in mammals (such as Human, Bovin, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster) Fig-8.

3.6. Phylogenetic tree.

Phylogenetic analysis was conducted which involved the identification of homologous sequences, their multiple alignments, the phylogenetic reconstruction and the graphical representation of the inferred tree. On the Phylogenetic tree, we have a plot motif of GAPDH which is **[IL]-x-C-x-x-[DE].** [7] This is a GAPDH motif that consists of 6 amino acids **LTCRLE**. This motif is responsible for signal transduction and cell function induce transglycosylase activity shuttling of nitric oxide from nitric oxide synthase to Target protein. Where it is present only in 7 organisms among them 6 are mammals and 1 is a parasite. The bootstrap support values of these branches are 0.99. We deduce that signal

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transduction and cell function induce transglycosylase activity shuttling of nitric oxide from nitric oxide synthase moonlighting function evolved exclusively in the Mammalian branch. It is interesting to note that Haemoncus contorts a parasite which is a nematode worm that also contains the same motif. present in the mammals and the sequence is much closely related to all the mammals. We hypothesise that this may be due to a horizontal gene transfer of the GAPDH gene between the host which are ruminants and the nematode. It will be interesting to test this further.

4. DISCUSSION

The research and our findings show how some of the moonlighting functions due to post-translational modifications evolved in a highly conserved enzyme-like GAPDH. Post-translational modification(PTM) is an important means of regulating the functions of proteins. Some of the moonlighting functions are regulated by PTMs which regulate the binding partner of the proteins affecting their function or sometimes a gain of function (here a moonlighting function).

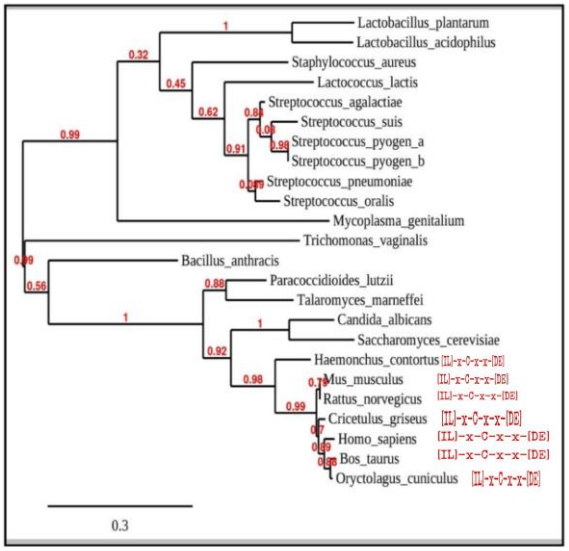

Fig:9

Here we emphasise how novel functions can evolve without any changes attributed to the sequence of the proteins but by simple post-translational modifications. We build evidence from multiple lines by tracing the presence of the amino acid residue that undergoes PTM, availability of the binding partner and the presence of the motif required for moonlighting function. acetylation, phosphorylation, S-nitrosylation bring about associated cell apoptosis. This PTM regulation is brought about by the motif[**IL]-x-C-x-x-[DE]** single motif found for GAPDH which helps in translocation of the Nitric oxide to the targeted site, during the stress condition of the cell Nitric oxide are translocated into the cell by motif and the nitric oxide initiate the binding of the amino acid at post-translational amino acid sites with their binding partners which bring about the modification (acetylation, phosphorylation, S-nitrosylation) needed to carry out Apoptosis. Further in the list of our selected organs, almost all have 7 organisms which are animals. Six are mammals (Human, Bovin, Rabbit, Mouse, Rat, Cricetulus griseus (Chinese hamster) and one is parasite Haemonochus contortus (commonly known as barber's pole worm) PTM conserved within them but due to the lack of motif[**IL]-x-C-x-x-[DE]** in them these functions are not present in them. By the phylogenetic, we found that this motif [**IL]-x-C-x-x-[DE]** is present only in higher organisms such as mammals. And in single parasites which have evolved from the same branch. PTMs also play a role in the death and survival of cancer cells [22]. There are also several functions identified which are associated to the GAPDH as moonlighting proteins. It will be interesting to see how these functions evolved and contributed to the complex functioning of GAPDH.

5. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. Authors confirmed that the pa-per was free of plagiarism

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