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Original Article

Insilco functional domain characterization and phylogenetic analysis of alpha 2 macroglobulin related molecule (α2M_SO) from *Spongia officinalis*

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ABSTRACT

A high molecular weight (182 kDa) serum protein, cardiac isoform of α 2 macroglobulin, functions as a protease inhibitor, minimizing cellular damage due to inflammation during cardiac hypertrophy. Based on the biological functions, it was speculated that this protein could have its origin from a primitive metazoan like sponge in response to immune and environmental challenges. This uncertainty prompted us to seek for an alpha 2 macroglobulin related protein in the poriferan, marine sponge, Spongia officinalis. To our surprise, RT PCR for the sponge total RNA with primers for 182 kDa rat serum protein produced a 738 bp amplicon with partial regions homologous to amino terminal (N region) and carboxy terminal (Receptor Binding Region), lacking other functional domains of rat 182kDa cDNA. We cloned and expressed the sponge cDNA for alpha 2 macroglobulin related molecule (α2M_SO) in E.coli and the synthesis of full length protein (~25 kDa) was confirmed with SDS-PAGE analysis. Computational analysis was performed to understand the structural and evolutionary context of the sponge protein. The deduced amino acid sequence contained two signature domains of alpha 2 macroglobulin protein. The three dimensional models predicted by homology modeling for α2M_SO confirmed its role in immunity as a protease inhibitor. Further phylogenetic studies suggested that the evolution of cardiac isoform of α 2 macroglobulin 182 kDa serum protein may have been forced to structural selection that results in genetic modifications leading to gradually highly complex cardiac hypertrophy specific 182kDa serum protein of enhanced stability and diversified functions in higher metazoans. This study thus revealed the existence of an alpha 2 macroglobulin related molecule (α2M_SO) in sponge and traces back to the origin of cardiac isoform of α 2 macroglobulin 182 kDa serum protein.

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1. Introduction

Cardiac enlargement refers to an increase in the size of the heart. There are two types of cardiac enlargement: hypertrophy and dilation. (Though usually occurring separately, they may occur at the same time.) Hypertrophy involves an increase in the thickness of the heart muscle. Dilation involves an increase in the size of the inside cavity of a chamber of the heart [1].

The 182 kDa serum protein, cardiac isoform of liver α-2-Macroglobulin found to play a critical role in cardiac hypertrophy and expression of muscle-specific genes in the heart [2]. It is presumed that increase in 182 kDa protein may act as a defensive mechanism in the implicated free radical damage that may occur in these cases. The 182 kDa serum protein, being a cardiac isoform of alpha-macroglobulin is known to be a powerful protease inhibitor

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may function as an effective antioxidant protein by influencing the rate limiting mechanism in the conversion of xanthine dehydrogenase to xanthine oxidase and thus inhibits its conversion [3].

The physiological role of α 2 Macroglobulin and its cardiac isoform (182kDa protein) [4], stimulated us to find the extent of its diversity and range of its functions in primitive organism such as Sponges. Sponges (Phylum:Porifera) are the most ancient and simplest extant multicellular organisms, branched first from the common ancestor of all metazoa. Since the genome sequence of demosponge S. domuncula is available, it could be useful in probing the origin of a particular protein [5].

Sponges represent large number of proteins that shares high sequence similarity with higher metazoan phylum which establishes the view of monophylectic origin of all metazoans [6]. Sequence data analysis in various sponges such as Geodium cydonium, Suberites domuncula [7], have high sequence homology with G–protein linked transmembrane protein [8], adhesion molecules with receptor tyrosine kinase [9], scavenger receptor cysteine-rich receptor [10], Vertebrate βγ crystallins [11] and integrin β subunit (12) respectively.

In this work, we showed the presence of an alpha 2 macroglobulin related molecule (α2M_SO) in the marine sponge Spongia officinalis . Further we investigated its structural properties and evaluated the extent of their conservation with the mammalian homologue (rat 182 kDa serum protein).The structural and phylogenetic analysis of α2M_SO among representative species uncovered varying degrees of conservation and evolution into gradually larger-sized proteins of improved and diversified functions related to alpha 2 macroglobulin protein family.

2. Materials and Methods

2.1.Collection and maintenance of Sponge

Spongia officinalis (Porifera, Desmospongiae, Keratosida, Spongiidae) were collected from the Sea near Karankadu (India), from 10m depth at about 16°C and transported in sea water within 4h and stored in liquid nitrogen until use.

2.2.RT-PCR, Cloning and Expression of α2M_SO

The total RNA was prepared [13] and the amount of RNA was quantified spectrophotometrically at 260 nm and purity checked by A260/280 ratio. RT-PCR was performed with the suitable thermal cycler program [14]. The PCR product (738bp) was cloned into pTZ57R/T vector (T:A cloning). An KpnI – SalI fragment of the cloned DNA was subcloned and expressed [14] with Bacterial Expression Vector pET32a (+) (Fermentas, USA). The recombinant protein of expected size (~25kDa) was visualised using SDS-PAGE.

2.3.GenBank Information

The nucleotide sequence is deposited in the GenBank database with the assigned Accession Number GQ353346 for the alpha 2 macroglobulin-related protein.

2.4.Sequence Analysis of α2M_SO

The protein sequence of the α 2Macroglobulin was deduced using BLASTX [15]. Similarity search was performed to identify significant homologues using NCBI_BLAST server, EBI-FASTA server [16] and PFAM database [17]. The presence of conserved domains and motifs were detected by performing multiple sequence alignment of the α 2 Macroglobulin with their significant homologues using EBI-CLUSTAL W [18] tool. The structural analysis of the α 2Macroglobulin was performed at varying hierarchy. The primary structural analysis was performed using EXPASY-PROTPARAM [19]. Folding of primary sequence into regular secondary structure of the protein was obtained using JPRED [20].

2.5.Molecular Modeling and Phylogenetic Tree Inference for α2M_SO

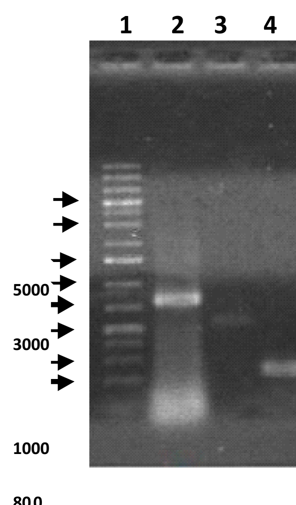
3D structure was modeled using Digital Studio Version 2.0.Template selection was done using BLASTp against PDB database [21] based on e-value & sequence similarity, PHYRE [22] and Conserved Domain database [23]. DOPE score analysis was performed and the quality of the model was analysed by PROCHECK [24]. The extent of stability of the secondary structure to elucidate the 3D model was carefully analysed. Distance matrix obtained through CLUSTAL W analysis was used to construct phylogenetic tree using MEGA [25]. The "goodness of the tree" was assessed by Bootstrapping.

3.Results and Discussion

We recently investigated the occurrence of a mammalian (rat) 182kDa serum protein related molecule in an oldest metazoan Spongia officinalis , to unravel the evolutionary origin of this physiologically significant protein.

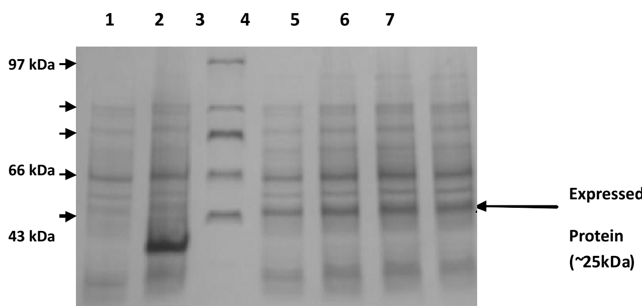
As described in the earlier work by Sivagama sundari et al., 2011[26], degenerate PCR primers were used to isolate homologous regions for 182 kDa serum protein cDNA from Spongia officinalis total RNA extract. Interestingly, the PCR product obtained was found to be a low molecular weight cDNA (738bp) (lane 4, Fig. 1) compared to the high molecular weight (4561 bp) of rat 182 kDa cDNA. Sequence analysis by BLAST showed highly homologous regions for N terminal (215bp) and receptor binding region (523bp) to rat 182 kDa cDNA (Fig. 1) with (99.9%) sequence similarity lacking other functional domains. The sequence for the alpha 2 macroglobulin related molecule (α2M_SO) in Spongia officinalis was deposited in GenBank with nucleotide accession number (GQ353346)

FIG 1: PCR Amplification of n terminal and receptor binding domain from spongia officinalis



PCR amplification of N terminal and Receptor Binding domain from Spongia officinalis using primers specific for 182kDa cDNA. Lane (1): 1kb plus DNA Marker; Lane (2) amplification of N terminal at 215bp; Lane (3) amplification of Receptor Binding domain at 523bp; Lane (4) amplification of full length cDNA from Spongia officinalis using forward primer of N terminal and reverse primer of RBR specific for 182kDa cDNA.

FIG 2. Expression of alpha 2m related molecule from *spongia officinalis.*



Expression of alpha 2M related molecule from Spongia officinalis: NaDadSo4 PAGE gel stained with coomassie Brilliant Blue showing the expression of the recombinant protein; lane (1) total lysate before induction with IPTG, lane (2) lysate obtained from cells without Insert (Vector alone) after IPTG induction for 5 hours, lane (3) molecular weight protein marker (Genei), lanes: 4, 5, 6&7 total lysate obtained form cells with insert (Vector + Insert) after IPTG induction for 2,3,4,5 hours respectively.

The full length sponge cDNA was cloned in to an [pET32a (+)] for E.Coli and the SDS-PAGE analysis confirmed the molecular mass of the target protein as ~25 kDa (Fig 2). Analysis of the deduced aminoacid sequence from BLASTX (Fig 3a) using PFAM displayed the presence of two conserved domains namely N terminal MG1 domain from α2Macroglobulin [pFam11974] and α2M receptor [pfam07677], which includes the receptor domain region of the alpha-2-macroglobulin family respectively (Fig 3b). It is assumed that during the course of evolution, additional sequences may have added that has led to the presence of a larger protein like 182kDa protein, an isoform of α 2 Macroglobulin, in the animal kingdom that have an important role in cardiac adaptation to mechanical load [1].

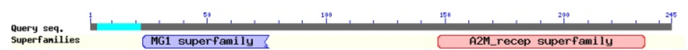
FIG (3a): Amino acid sequence deduced using blast X

1 gkhrlrslal lplllrllll llptdasapq kpiymvmvps llhagtpeka cflfshlnet
61 vavrvslesv hgrvtlptvp gdytvkvtge gcvylqtslk ysvlpreeef pftvvvqtlp
121 gtcedpkaht sfqislnisy tgsrsesnma iadvkmvsgf iplkptvkml ersvhvsrte
181 vsnnhvliyl dkvsnqtvnl sftvqqdipi rdlkpavvkv ydyyekdefa vakysapcst
241 dygna

Amino acid was deduced from the sequenced nucleotide by outsourcing using BLASTX.

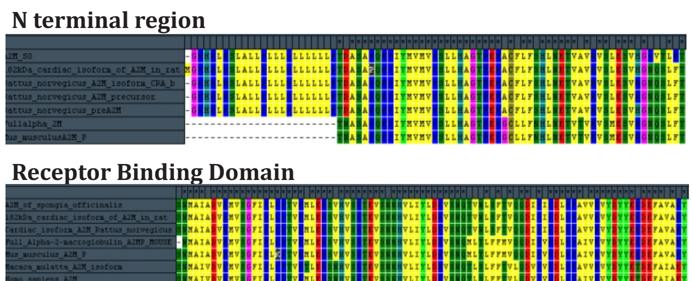
BLASTp showed maximum similarity with different isoforms of alpha 2 Macroglobulin in higher vertebrates and C3 complement factors. The Multiple sequence alignment of the retrieved amino acid sequences was done in CLUSTAL W (Fig 4) separately for N terminal and Receptor Binding Domain of A2M related molecule in Spongia officinalis, with those of their corresponding homologues (Table 1) based on their 'e' value. Both the domains confer a high degree of conservation of amino acids with their corresponding homologues which substantiate the evolutionary origin of sponge protein.

FIG (3b): Functional domain analysis using pFAM



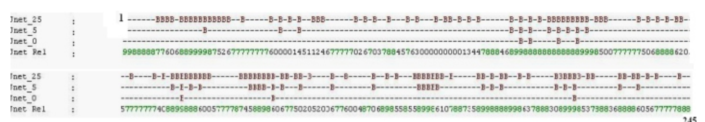
Conserved domain was analyzed using Blast X which displayed the presence of two domains which are N terminal and Receptor Binding Domain respectively.

FIG 4: Multiple sequence alignment for n terminal and receptor binding domain of *spongia officinalis* using clustal W



Multiple sequence alignment of alpha related molecule from Spongia officinalis was done using CLUSTAL W with their corresponding homologues represented in Table 1.

FIG 5: Secondary structure prediction using JPRED



Secondary structure prediction was performed using JPRED which conferred the conservation of amino acids during the course of evolution.

FIG 6a: Model of n terminal region

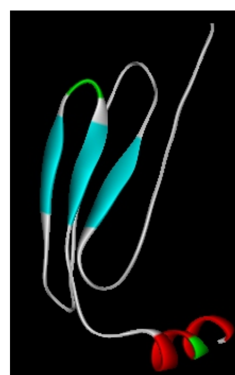
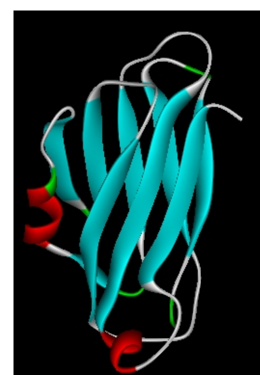


FIG 6b: Model of receptor binding region



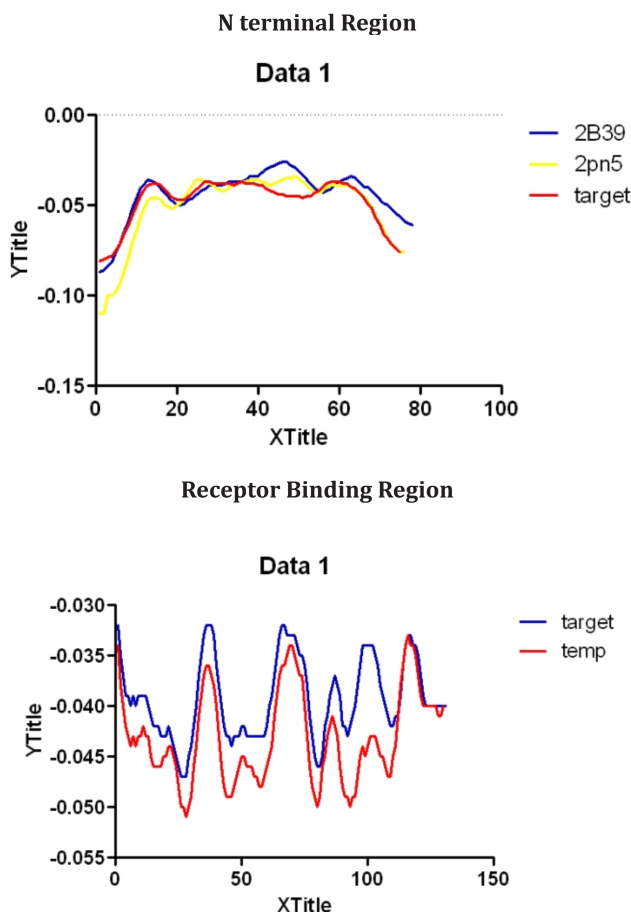
3 Dimensional modeling using Digital Studio Version 2.0 was done separately for N terminal and Receptor Binding Domain using appropriate templates that was selected from Protein Data

TABLE1: Multiple sequence alignment of *spongia officinalis* with their corresponding homologues

Accession Number	N Terminal	Accession Number	Receptor Binding Domain
ACY74611.2	Alpha 2 macrologulin-related protein *[Spongia officinalis]*	ACY74611.2	Alpha 2 macrologulin-related protein *[Spongia officinalis]*
J02635	182kDa cardiac isoform of liver alpha 2 macroglobulin *[Rattus norvegicus]*	J02635	182kDa cardiac isoform of liver alpha 2 macroglobulin *[Rattus norvegicus]*
AAA40636.1	Prealpha-2-macroglobulin *[Rattus norvegicus]*	AAW65786.1	Alpha 2 macroglobulin cardiac isoform *[Rattus norvegicus]*
NP_036620.2	Alpha-2-macroglobulin precursor *[Rattus norvegicus]*	Q6GQT1.1	Full=Alpha-2-macroglobulin-P *[Mus musculus]*
EDM02008.1	Alpha-2-macroglobulin, isoform CRA_b [Rattus norvegicus]	AAO25741.1	Alpha-2-macroglobulin-P *[Mus musculus]*
Q6GQT1.1	Full=Alpha-2-macroglobulin-P *[Mus musculus]*	XP_001114328.1	Alpha-2-macroglobulin isoform 3 *[Macaca mulatta]*
NP_783327.2	Alpha-2-macroglobulin-P precursor *[Mus musculus]*	AAT02228.1	Alpha 2 macroglobulin *[Homo sapiens]*

The Multiple sequence alignment of the retrieved amino acid sequences was done in CLUSTAL W separately for N terminal and Receptor Binding Domain of A2M related molecule in Spongia officinalis, with those of their corresponding homologues based on their 'e' value.

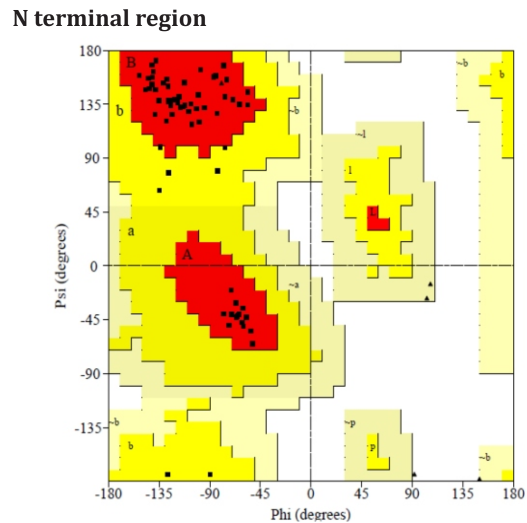
FIG 7: Discrete optimised protein energy (DOPE) score for best model prediction



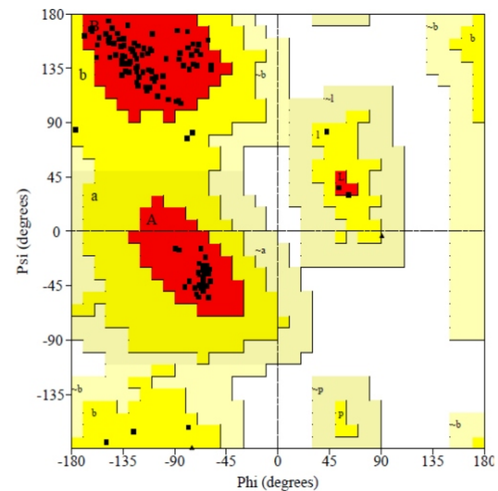
Graphical symbol of the relative stability of confirmation of the target protein structure with that of the template selected was done using Discrete Optimised Protein Energy (DOPE) score analysis.

FIG 8: Ramachandran plot using procheck analysis

N terminal region



Receptor Binding Region



Quality and the goodness of the model were checked by PROCHECK analysis using Ramachandran plot.

Fig 9: Phylogenetic analysis of alpha related molecule in spongia officinalis with their corresponding homologues using

N terminal Region

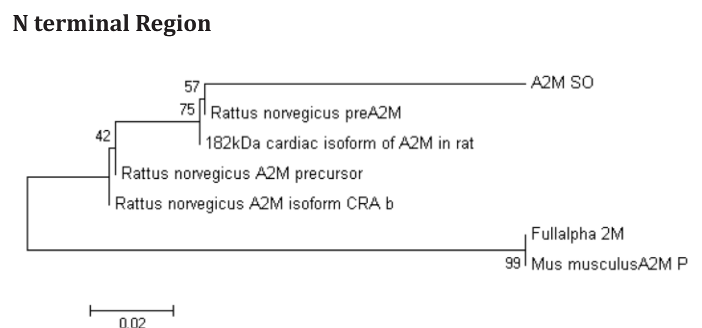
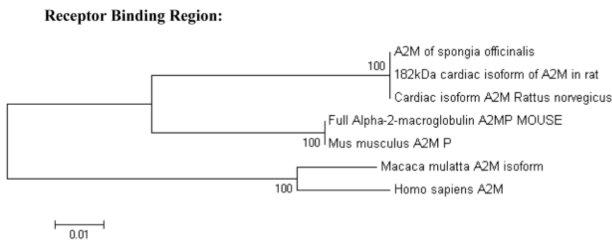


Fig 9: Phylogenetic analysis of alpha related molecule in spongia officinalis with their corresponding homologues using



Evolutionary origin of the alpha related protein from *Spongia officinalis* was assessed by phylogenetic analysis with their corresponding homologues using MEGA software.

Analysis of Physico-chemico parameter using EXPASY–PROTPARAM tool, revealed that the S.officinalis protein has an instability index of 37.96, classifying it as a Stable protein. The molecular weight of protein is calculated to be ~27141.4 and the theoretical pI as 7.83. Half life of the protein is estimated to be 30 hours in mammalian reticulocytes, invitro, >20 hours in yeast, invivo,>10 hours in Escherichia coli, invivo. Grand average of hydropathicity is (GRAVY): 0.033.

Secondary structure prediction was done using JPRED tool (Fig 5) using three different algorithms such as Jnet, Jhm and Jpsmm. The conserved pattern based on these algorithms was represented in Fig 2. The conservation of chemical nature of aminoacid was found to be higher which suggests that this particular α2M_SO related protein have conserved amino acid which diversifies later into more complex protein in higher vertebrates.

3D model was constructed using Digital Studio Ver2.0. Modeling was done separately for individual domains (N terminal and Receptor Binding Domian). N terminal region was modeled using Threading (Fig 6a). Template was selected from PHYRE and Conserved Domain Database. 2B39 (Structure of mammalian C3 with an intact thioester) and 2PN5 (Crystal Structure of Thioester-containing proteins TEP1r) has similar secondary structure pattern with that of the target sequence, hence used as a template for modeling N terminal region. The template was found to be the major component of the innate immune response of lower organisms to invasion by bacteria and protozoans [27]. Similarly Template for Receptor Binding Region was done using BLASTp against PDB. 1AYO (receptor binding domain of bovine alpha-2-macroglobulin) was selected as template for Receptor Binding Region (Fig 6b). It is the large plasma proteinase inhibitor of the α2 Macroglobulin superfamily which inhibits proteinases by forming α2 M – Proteinase complex that binds to α M receptor in liver and other tissues, becomes endocytosed and rapidly removed from the circulation [28].

The best one out of five different models was chosen based on the least DOPE Score value for both N terminal and RBR of C terminal which is found to be – 4618.46 and – 13975.12 respectively. Fig 7 represents the graphical symbol of the relative stability of confirmation of the target protein structure with that of the template selected.

The quality of residue backbone conformations in 3D model of the protein was assessed by Ramachandran plot using PROCHECK. As shown in Fig 8 the quality of the Ramachandran plot as well as the goodness factors was found to be better for the 3D model of both domains of Spongia officinalis. The distribution of the ψ/Φ angles of the models in the most allowed region (88.9% & 94.1%), in additional allowed region (11.1% & 5.9%), and 0% in both generously allowed region and in disallowed region. Thus, the above analysis suggests the backbone conformations of 3D Model of the protein found to be better, suggesting the 90% probability of having good quality model.

Rooted phylogentic tree of the Spongia officinalis α2M_SO was constructed by using MEGA software by Neighbour-Joining (Fig 9) after a multiple sequence alignment (CLUSTAL W program) with the corresponding sequences of α2M isoforms. In order to understand the evolutionary origin of the α2M_SO Protein, a narrow range of animal phylogeny was made which revealed that all the Metazoans are of monophyletic origin [28].

As discussed by Brower et al., 1997 [29] although the major structural features of integrin β subunit are conserved in corals and sponges, there is no indication that they are specialized proteins such as vertebrate β1 and Drosophila βv. That is, the sequences represent major tissue integrins of these organisms in the same way that vertebrate β1 and Drosophila βPS are common in their respective organism. In a similar manner, sponge related alpha 2 Macroglobulin may also have the essential structural genes conserved as N terminal and Receptor Binding region which may be involved in signal transduction pathways in such an oldest metazoan phyletic lineage.

The alpha 2 Macroglobulin related protein in Vertebrates have diversified to establish additional functions that likely relate to the large changes that occurred during chordate evolution in the transition to cartilaginous and bony internal structures, a dual innate and adaptive immune system and the high-pressure circulatory system that characterize the vertebrate lineage[30]. Thus, the vertebrate alpha 2 Macroglobulin protein did not appear suddenly, but arose from homologous sequences and domains that pre-existed their functions in cell signaling or may have immune function in differentiating self and non-self in the primitive metazoans and existing within the protists and prokaryotes.

4.Conclusion

Our study provides a detailed insight into the evolutionaryorigin of 182 kDa rat serum protein, cardiac isoform of α 2 macroglobulin. In silico analysis of the alpha 2 Macroglobulin related protein revealed regions homologous to 182 kDa rat serum protein that may be have similar kind of functions as in higher vertebrates. Recently, complete genome sequencing of a particular sponge Amphimedon Queenslandica was published which reveals genomic events linked to the origin and early evolution of animals, including the appearance, expansion and diversification of pan-metazoan transcription factor, signaling pathway and structural genes [31] Also the presence of N terminal and C terminal may be involved in signalling pathways that helps in cell to cell recognition which is similar to antibody antigen recognition with theinvolvement of cell surface proteins. Based on our findings we

conclude that the molecular evolution of larger 182kDa protein from the primitive protein (α2M_SO) must have occurred in a species such as Sponges that was a direct ancestor of all the existing vertebrates.

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Abbreviations:

α2M_SO - Alpha 2 macroglobulin related molecule from spongia officinalis, RBR – Receptor Binding Region, DOPE – Digital optimized protein Energy score, RT PCR – Reverse Transcriptase Polymerase Chain Reaction, SDS-PAGE –Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis

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