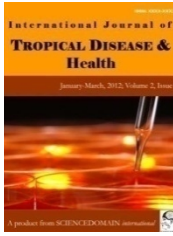

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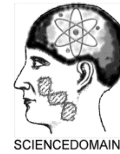

Identification of the N-terminal Glycine-arginine Rich (GAR) Domain in *Giardia lamblia* Fibrillarin and Evidence of its Essentiality for snoRNA Binding

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SK and DR carried out the necessary bench works and drafted the manuscript. Author SG designed the study, analysed the data and wrote the paper. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Study the role of glycine-arginine rich (GAR) domain of fibrillarin in *Giardia lamblia*.
Study Design: Identifying a specific glycine arginine rich (GAR) sequence of *Giardia* fibrillarin which plays an important role in ribonucleoprotein particles complex formation with snoRNA during post transcriptional modifications of rRNA. The present study uses Electrophoretic Mobiliy Shift Assay (EMSA) to detect protein-RNA interactions. ^{32}P labeled snoRNA incubated with purified fibrillarin or GAR domain truncated fibrillarin protein were separated by a non denaturing polyacrylamide gel where the band patterns suggested the interaction of the RNAs with both proteins.
Place and Duration of Study: Department of Parasitology, National Institute of Cholera and Enteric Diseases, Indian Council of Medical Research, Kolkata, between January 2011 and July 2012.
Methodology: Homology analysis of *G. lamblia* fibrillarin was performed with fibrillarins from *Homo sapiens*, *Mus musculus* and *Arabidopsis thaliana* to determine the conserved domain by ClustalW analysis. Cloning, expression and purification of fibrillarin and GAR domain truncated fibrillarin were done to study the role of GAR domain in snoRNA-

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fibrillarin binding by EMSA and Gradient EMSA. Immunoblot assay of purified GAR domain truncated fibrillarin was performed by using polyclonal antibody of purified recombinant giardia fibrillarin protein.
Results: Gel retardation assay showed that snoRNA does not bind with GAR domain truncated fibrillarin to form any RNP complex. Similarly in gradient EMSA the GAR domain truncated fibrillarin does not bind with any of the *in vitro* transcribed snoRNA even at much higher concentration than they do for full length purified recombinant fibrillarin.
Conclusion*:* The amino terminal conserved glycine arginine rich (GAR) domain is present in *Giardia lamblia* fibrillarin and is essential for binding with snoRNA of this protein.

Keywords: Giardia lamblia; small nucleolar RNA; fibrillarin; RNA-Protein complex; glycine-arginine rich domain.

1. INTRODUCTION

Giardia lamblia (also known as *Giardia intestinalis* and *Giardia duodenalis*) is an amitochondrial, unicellular, gastrointestinal flagellated protozoan parasite causing one of the most common parasitic infections worldwide [1]. It imparts to an estimated 300 million symptomatic human infections (called Giardiasis) per year and has been included as part of WHO Neglected Disease Initiative since 2004.

The diplomonad protist *Giardia lamblia* is a prevalent human enteric parasite that displays a highly reduced compact genome and somewhat limited metabolic capacity [2]. Eukaryotic precursor (pre-) RNA processing often requires ribonucleoprotein (RNP) complexes consisting of conserved and essential non-coding (nc) RNAs. Notable examples are the small nucleolar (sno) RNPs that participate in eukaryotic ribosome biogenesis through structural modification of specific nucleotides in ribosomal RNA (rRNA) and/or targeting cleavage of the pre-rRNA [3]. Although it was previously considered that *Giardia* lacks a nucleolus, recent studies have reported the organism to be nucleolate [4].

It has been demonstrated recently that numerous small nucleolar RNAs (snoRNA) found in the nucleolus of eukaryotic cells, can be sorted into two classes [5]. Both of them are involved in pre rRNA processing. Members of one class containing structural motifs called box C/D are involved in 2’-O-ribose methylation of pre rRNA. The other class of snoRNAs are characterized by the conserved motifs called box H/ACA which converts the Uridine (U) residue of the pre rRNA into pseudouridine (ψ) [6,7]. Recently, 20 snoRNA-like RNAs have been identified in *Giardia* out of which 16 belong to C/D box class and the rest are H/ACA type [8]. Fibrillarin (Fb) is a methyl transferase [9] that is required for pre rRNA processing. Association of C/D box snoRNA with fibrillarin has been reported previously [10,11] and in *Giardia*, the protein associates with RNA D, RNA J, RNA H, both *in vitro* and *in vivo* [12]. Deletion analysis of fibrillarin in other organisms indicated that a major RNA binding element, which is extremely well conserved throughout evolution, lies in the amino terminal end of the protein [13]. The sequence is highly conserved between different species and confirms the previously observed similarity between different mammalian nucleolar proteins. The motif is approximately 80 residues long and rich in glycine and arginine (GAR domain) [14]. A model of box C/D snoRNP assembly proposes that this conserved GAR domain is necessary for fibrillarin to bind snoRNA. The domain carries the pre-rRNA base-pairing interactions in the functional center of the U3 snoRNP [15].

It has been proposed that specific interaction of snoRNA with this amino terminal domain of fibrillarin in the nucleolus occurs for pre-rRNA processing [16]. Here in this study we describe the identification of the GAR domain of *Giardia lamblia* fibrillarin protein and the essential role it plays for binding snoRNAs. This is the first demonstration of the importance of GAR domain in *Giardia* snoRNA binding for rRNA processing complex formation.

Understanding of the cell biology of *G.lamblia* will greatly increase our knowledge about this organism as well as promise to yield novel insight into the evolution of key pathways and structures of higher cells. Thus, what commenced as an interest in the molecular biology of medically important protozoa has developed into a fascinating expedition through the unusual eukaryote, *Giardia*.

2. MATERIALS AND METHODS

2.1 Homology Analysis to Determine a Conserved N Terminal Glycine Arginine Rich Domain in *G. lamblia* Fibrillarin

Homology analysis of *G. lamblia* fibrillarin was performed with amino acid sequence of fibrillarins from *Homo sapiens*, *Mus musculus* and *Arabidopsis thaliana* to determine the conserved domain. The analysis was performed by ClustalW using SeaView Graphical representation version 4.

2.2 Cloning of Fibrillarin (pET33b-Fb) and N Terminal GAR Domain Truncated Fibrillarin (pET33b-δGFb)

The *Giardia* fibrillarin gene was cloned in pET 33b vector as described previously [12]. For the cloning of pET33b-δGFb two primers were designed, with restriction sites engineered into them that precisely excluded the GAR domain of the protein (Table 1). The desired fragment was PCR amplified from *Giardia* cDNA and cloned into pET 33b vector. Briefly, the PCR products and the plasmid were digested with BamHI and NdeI restriction enzymes, purified and then ligated using T4 DNA ligase at 16ºC overnight. After the reaction was over, the enzyme was inactivated at 65ºC for 10 mins. The ligation mixture was transformed into competent *E.coli* XL1 Blue cells. Positive clones of GAR domain truncated fibrillarin were selected randomly from LB plates containing 50µg/ml Kanamycin. The plasmid isolated from each selected clone was double digested by BamHI and NdeI restriction enzymes, separated on a 1% agarose gel and photographed.

Table 1. Primers for PCR amplification of GAR domain truncated fibrillarin gene of *Giardia lamblia*

Name	Sequence	Restriction enzyme site
GAR(TFb)_F	5'-C**GGATCC**GAUGCGGACCAAGGG-3'	BamHI
GAR(TFb)_R	5'-GGAATTC**CATATG**GAATTCCTCACGCTGCCTTG-3'	NdeI

2.3 Recombinant Proteins Expression and Purification

Recombinant fibrillarin gene was expressed in *E.coli* BL21 (DE3) cells and purified as explained previously [12]. For bacterial expression of GAR domain truncated fibrillarin gene, pET33b-δGFb was transformed to *E.coli* BL21 (DE3) cells. These cells were grown in LB

supplemented with 50 µg/ml kanamycin and 0.3% glucose and incubated with shaking at 25ºC until OD_{600} reached 0.6, followed by 1 mM IPTG (Sigma) induction. The cells were induced for 5 hr and harvested by centrifugation at 12,000g for 10 mins at 4ºC. The pellet was washed with PBS and resuspended in 2 ml of ice-cold binding buffer (20mM Tris-HCl, pH 7.9, 5mM imidazole and 0.5 M NaCl) then it was dissolved in 1 ml lysis buffer (10X lysis buffer - 0.5 M Tris-HCl (pH 8.0), 10 mM EDTA, 1M NaCl) and sonicated in an ultrasound sonicator by 10-15 30-seconds bursts at 20 kilocycles at an interval of 1 min at 4ºC. Sonicated material was centrifuged at 12000 g for 10 min at 4ºC to remove the debris and the supernatant was used for purification. Purification was done using His tag column (HiTrap Chelating®, Amersham Pharmacia) equilibrated with $NiSO_4$ and binding buffer as per manufacturer's protocol.

The crude soluble GAR domain truncated fibrillarin protein was loaded onto this pre equilibrated His Tag column. The unbound proteins were washed with 10 column volumes of binding buffer with 5mM imidazole and then with 6 column volume of wash buffer containing 20mM imidazole. The target protein was eluted with 6 volumes of elution buffer [20mM Tris-HCl (pH7.9), 250mM imidazole and 0.5M NaCl]. Subsequently the imidazole and NaCl was dialyzed out against dialysis buffer (10 mM Hepes-KOH, pH 7.9, 10% glycerol, 0.5% NP-40, 1 mM EDTA, pH 8.0, 0.5 mM DTT, 100Mm KCl, and 5mM $MgCl_2$) using centricon (pore size 10000 daltons). The purified protein fractions were analyzed by SDS-PAGE.

2.4 Western Blot Analysis

Purified GAR domain truncated fibrillarin and *E. coli* crude soluble antigen (CSA) (prepared in the laboratory) were separated by 10% SDS PAGE. Polypeptides from this SDS PAGE were transferred onto nitrocellulose membranes (pore size 0.45 µm, Bio-Rad, USA) using a Trans-blot apparatus (Bio-Rad, USA) and transfer buffer (1.162gm Tris-HCl, 0. 0585gm glycine 0. 075gm SDS in 160ml of water and 40ml of methanol) at a constant voltage of 100 volts for 2 hours at 4ºC. Nitrocellulose strips were washed thoroughly in Tris buffered saline (25mM Tris-HCl (pH 7.4), 150mM NaCl) and incubated with blocking buffer (3% BSA in TBS) at 4ºC overnight. The nitrocellulose strips were washed with TBST (TBS + 0.05% Tween 20) and incubated with anti-Giardia fibrillarin (developed in rabbit) 1:50 dilution, at 37ºC for 90 minutes on a rotatest shaker. The strips were washed again (as above) and incubated with anti-rabbit IgG-HRP (Horseradish peroxidase) (Jackson Immunoresearch Lab, USA), 1:5000 dilution. at room temperature for 45 minutes. The washed nitrocellulose strips were developed in 0.05% solution of 3,3'- diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) and 0.03% hydrogen peroxide in TBS. The reaction was allowed to proceed in darkness for 4 to 5 minutes and was stopped by repeated washing in distilled water.

2.5 Electrophoresis Mobility Shift Assay (EMSA)

40,000cpm ^{32}P labeled snoRNA and 2µg purified GAR domain truncated fibrillarin protein were mixed with binding buffer (10mM HEPES-KOH pH 7.9, 10% glycerol, 0.05% NP40, 1mM EDTA pH 8.0, 0.5mM DTT, 100mM KCl and 5mM $MgCl_2$). The total reaction volume was 20 µl and the reaction mixture was incubated at 0ºC for 30 min. After incubation samples were electrophoresed on a native 5% polyacrylamide gel (29:1) in TAE buffer at 100 volts at 4ºC. Different control reactions were also performed using full length recombinant fibrillarin protein, Bovine Serum Albumin (BSA), labeled RNAs both specific (RNA J and RNA H of *Giardia*) and non-specific (a 82 nucleotide leader RNA of Chandipura virus, a negative strand RNA virus).

2.6 Gradient Electrophoresis Mobility Shift Assay (GEMSA)

20µL binding reaction contains binding buffer (10mM HEPES-KOH pH 7.9, 10% glycerol, 0.05% NP40, 1mM EDTA pH 8.0, 0.5mM DTT, 100mM KCl and 5mM $MgCl_2$), 40,000cpm ^{32}P labeled snoRNA (RNA D) and different amount (i.e. 10-100ng) of purified GAR domain truncated fibrillarin protein. The reaction mixes were incubated at 0ºC for 30 min. After incubation samples were electrophoresed on a 5% native polyacrylamide gel (29:1) in TAE buffer at 100 volts and 4ºC. Positive control reactions using same concentrations of full-length recombinant fibrillarin protein and labled snoRNA were used.

3. RESULTS

3.1 Homology Analysis of Fibrillarin in *G. lamblia*

Homology analysis of *G. lamblia* fibrillarin sequence has revealed that N terminal Glycine Arginine Rich (GAR) domain of *Giardia lamblia* fibrillarin is largely homologus and conserved with fibrillarin from different sources, *viz. Homo sapiens, Mus masculus,* and *Arabidopsis thaliana* (Fig. 1).

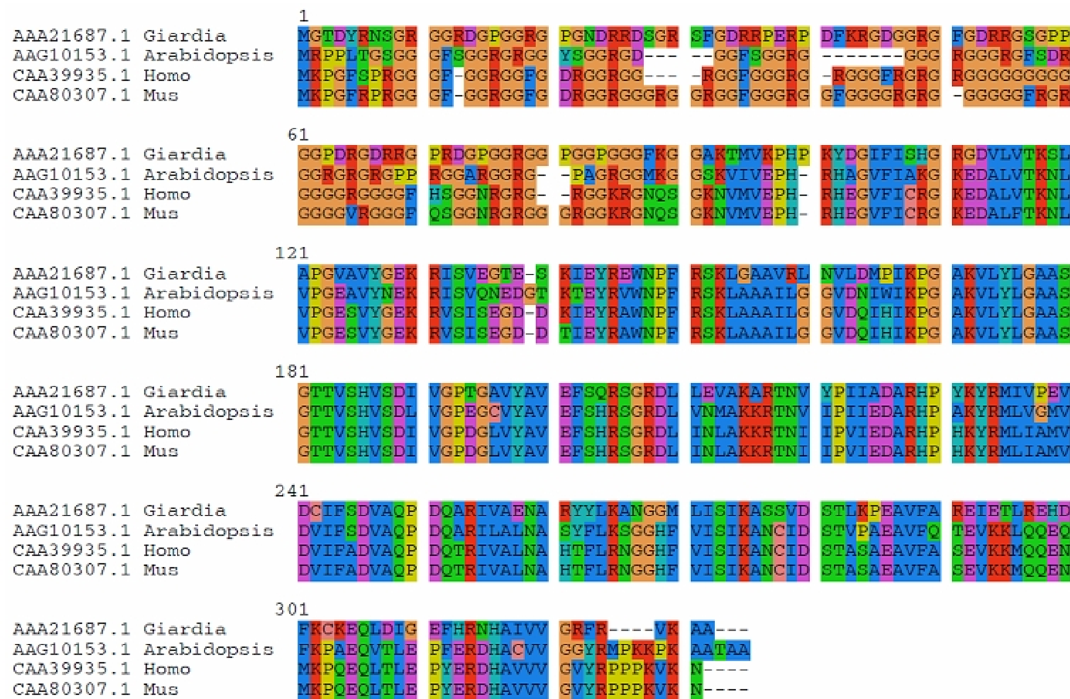

Fig. 1. Homology of amino terminal conserved glycine arginine rich (GAR) domain of *G. lamblia* fibrillarin with fibrillarin sequence from other organisms

3.2 Expression and Purification of Recombinant GAR Domain Truncated Fibrillarin Protein

Purified fraction of recombinant GAR domain truncated fibrillarin and wash outs were run in 10% SDS PAGE which reveals a distinct band of purified GAR domain truncated fibrillarin after staining with Coomassie blue (Fig. 2).

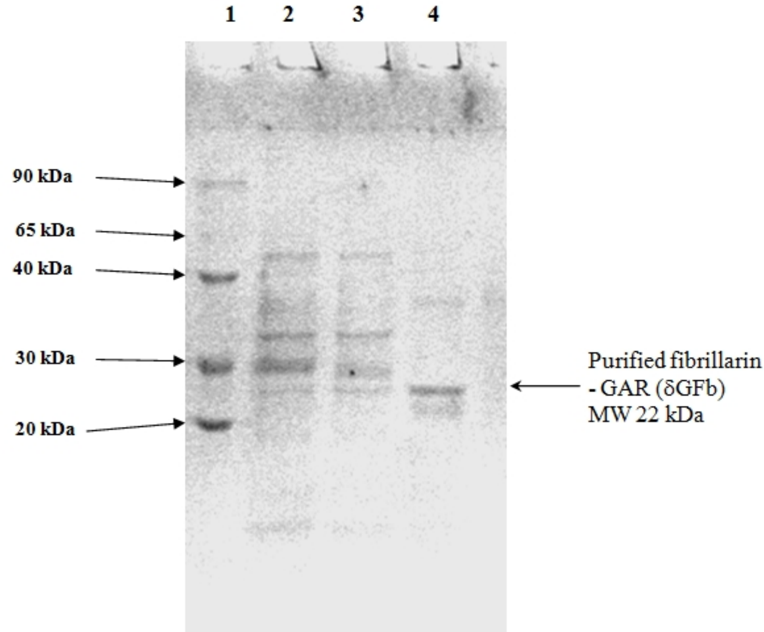

Fig. 2. Coomassie blue stained SDS PAGE showing purification of GAR domain truncated fibrillarin: Lane 1: protein molecular weight marker, Lane 2 & 3: fractions containing wash solutions. Lane 4: fraction containing purified GAR domain truncated fibrillarin

3.3 Western Blot Analysis

Immunoblot assay of purified GAR domain truncated fibrillarin has produced a distinct band with polyclonal antibody against full length purified fibrillarin (Fig. 3). This indicates that the 3 dimensional structures of the other domains of GAR truncated fibrillarin remains equally active as in full length fibrillarin. Thus, study with this protein will definitely explain the role of GAR domain in binding with snoRNAs as all other domains are active and could bind with the snoRNAs (if they possibly binds).

3.4 EMSA

Gel retardation assay showed that none of the three snRNA binds GAR domain truncated fibrillarin to form any complex (Fig. 4). However, full length purified recombinant fibrillarin that has been used as a positive control has showed clear shifting of band. Similarly, in gradient GEMSA the GAR domain truncated fibrillarin does not bind with any of the *in vitro* transcribed snRNA even at much higher concentration than they bind in case of full length purified recombinant fibrillarin (Fig. 5).

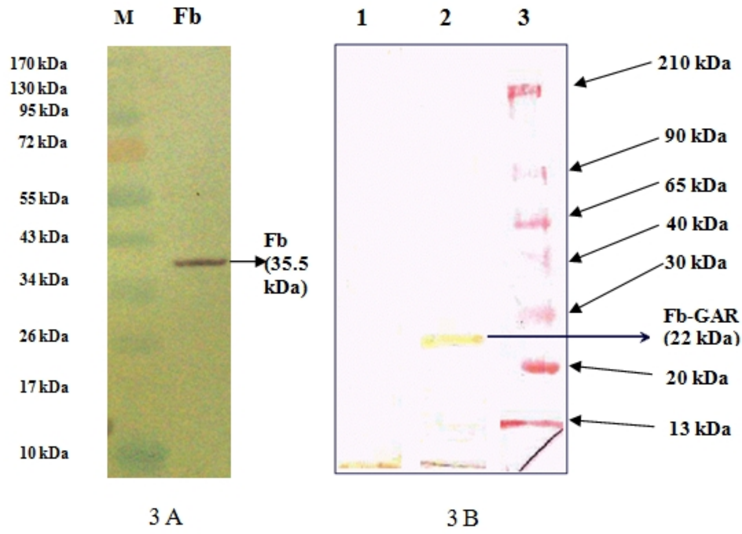

Fig. 3A. Western blot of recombinant purified fibrillarin protein of *Giardia lamblia*
Fig. 3B. Western blot of recombinant purified GAR domain truncated fibrillarin protein of *Giardia lamblia* Lane 1: E.coli CSA; Lane 2: GAR domain truncated fibrillarin; Lane 3: Protein molecular weight marker

RNA D	+	+	+	-	-	-	-	-	-	-	-	-	-
RNA J	-	-	-	+	+	+	-	-	-	-	-	-	-
RNA H	-	-	-	-	-	-	+	+	+	-	-	-	+
Fb	+	-	-	+	-	-	+	-	-	+	-	-	-
δGFb	-	-	+	-	-	+	-	-	+	-	-	+	-
BSA	-	-	-	-	-	-	-	-	-	-	-	-	+
Viral RNA	-	-	-	-	-	-	-	-	-	+	+	+	-

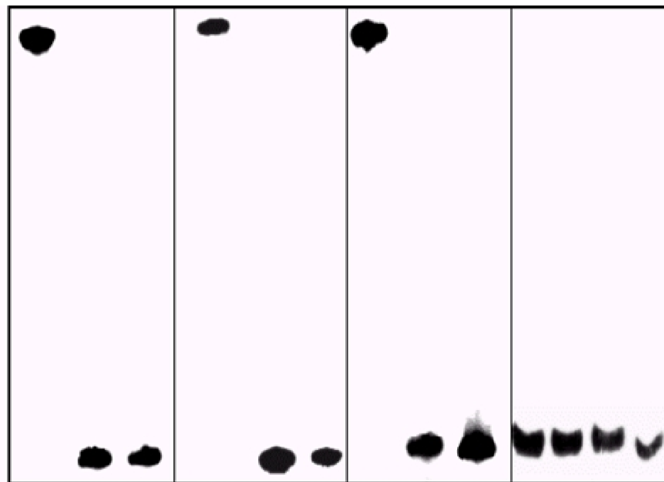

Fig. 4. Gel shift assay showing the interaction of *Giardia* snoRNAs with fibrillarin (Fb) and GAR truncated fibrillarin (δGFb) proteins. BSA is used as a non-specific protein and viral RNA is used as non-specific RNA to serve as negative controls for this experiment

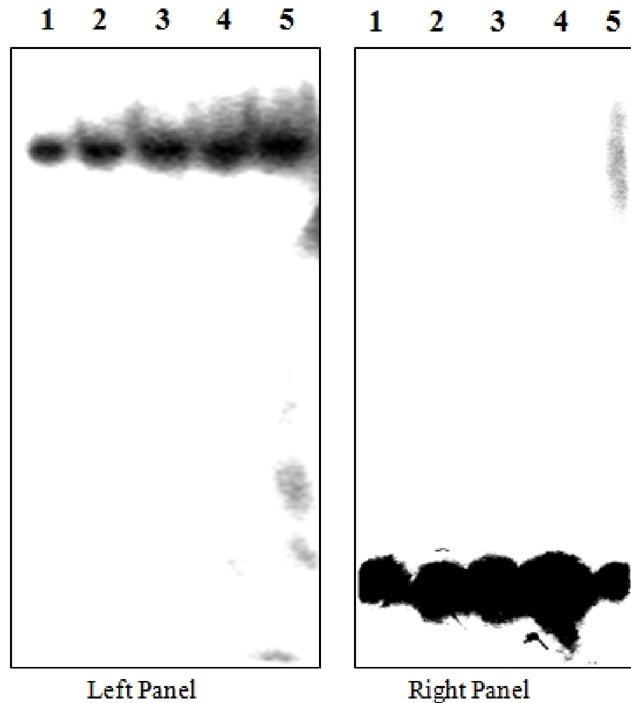

Fig. 5. Gradient GEMSA using increasing amounts of full length fibrillarin (left panel) and GAR domain truncated fibrillarin (right panel) incubated with snRNA D. Lane 1 : 10μg Fb, Lane 2: 20μg Fb, Lane 3: 40μg Fb, Lane 4: 80μg Fb and Lane 5: 100μg Fb

4. DISCUSSION

Fibrillarin (~35 kDa) comprises three structural domains, and is conserved among different organisms and species. Homology analysis of conserved domain in fibrillarin has suggested the importance of N terminal GAR domain. Fibrillarin has been identified in *Giardia* and it has been shown that it interacts with snRNA *in vitro* and *in vivo* in the formation of RNPP complex, but, how this interaction takes place and what are the binding domains for this complex formation has not yet been identified. Deletion analysis has revealed that the GAR domain contributes to the binding of snoRNA. In the EMSA study, absence of any proper shift clearly suggests the important role of this GAR domain in RNPP formation and maturation. This may be either due to the RNA binding role of the GAR domain or may be due to change in the activity of RNA binding domain due to absence of GAR domain.

5. CONCLUSION

Our findings clearly demonstrate that the GAR domain of *Giardia* fibrillarin protein is essential for the binding of this protein with its snoRNAs. This finding is of potential importance to our understanding of snoRNP biogenesis in *Giardia lamblia.*

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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