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Full Length Article

Cloning and Phylogenetic Analysis of RNA Helicase p68 Encoding *DDX5* Gene from Human MCF7 Cell Line

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Abstract

In this study, genomic DNA was isolated from MCF7 cells and used as template for polymerase chain reaction (PCR) to amplify p68 by using sequence specific primers. The PCR product was purified, ligated into pMSCV-GFP vector and transformed into *E. coli* DH5α competent cells. Restriction analysis was performed to confirm the cloning. Further, the gene was sequenced, and on the basis of this partial sequence, a phylogenetic tree was made to access homology among different organisms. In present study, gene encoding p68 in human was found to be more close to gene encoding p68 in *Sus scrofa* and *Pan troglodytes.* The results obtained from present study will be helpful to study the role of p68 in regulation of gene expression in humans in future. © 2014 Friends Science Publishers

Keywords: p68; RNA helicase; phylogeny; MCF7; Cloning

Introduction

RNA helicases is a big group of conserved proteins present in all groups of organisms. Cell development, cell division, cell differentiation, mRNA synthesis and processing, assembly of ribosome and protein synthesis are different cellular function affected by these enzymes. These are important for remodeling of ribonucleoproteins as they have the ability to disrupt interactions between protein and RNA (Tanner and Linder, 2001; Zhu and Ren, 2012).

DEAD-box (DDX) proteins, comprising of 38 members in humans is the biggest group of RNA helicases; presence of signature amino acid sequence (Asp-Glu-Ala-Asp, or D-E-A-D), which is highly conserved is the reason for this name. Manipulation of RNA structures is a vital role played by many proteins of DEAD box family. The presence of RNA, either double-or single-stranded, stimulates ATP hydrolysis, which is carried out by these DDX proteins, but in a few processes these DDX proteins act as true RNA helicase. DEAD box proteins act as ATP-dependent helicases, they disrupt interactions in RNA–protein complexes, which are involved in vital processes like degradation of RNA, transcription, mRNA processing, translation, RNA export from nucleus (Linder, 2006). Dysregulated expression and genomic amplification of RNA helicases (DEAD box) has implications in the development of cancer. Many of these proteins perform important functions in processes related to transformation or cellular proliferation. DEAD box proteins are the subject of interest in cancer research due to their roles in progression or development of cancer (Godbout *et al.*, 2007; Schroder, 2010).

p68 (*DDX5*) is a prototypic member of the DEAD box family (Ford *et al.*, 1988) and is an established ATPase and RNA helicase (Hirling *et al.*, 1989; Iggo and Lane, 1989). Previous reports have shown that p68 expression is growth and developmentally regulated and that p68 is overexpressed and abnormally polyubiquitylated in colorectal tumours (Stevenson *et al.*, 1998; Causevic *et al.*, 2001). *In vitro* experiment has shown p68 to be essential for splicing of pre-mRNA (Liu, 2002) and it participates in regulation of alternative splicing of c-H-ras (Guil *et al.*, 2003). It predominates in the cell nucleus due to its role in transcriptional regulation as well as pre-mRNA splicing. It is clear now that p68 moves between cytoplasm and nucleus as it also stays in cytoplasm for a short period of time. This movement between nucleus and cytoplasm takes place via RanGTPase-dependent pathway with the help of two nuclear localizaton and two nuclear exporting signal sequences (Wang *et al.*, 2009; Mustafa *et al.*, 2013).

Presence of p68 in all groups of living organisms has been reported previously but little is known about its evolution. Any knowledge about the evolution of this gene would be helpful to establish its role in other organisms as well. Therefore, present project was designed to isolate and clone *DDX5* gene from human and to study its phylogenetic relation with different organisms.

Materials and Methods

Maintenance of Cell Culture

MCF7 cells were cultured in Dulbecco's Modified Eagle

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Medium (DMEM glutMAx), supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in 5% CO_2 atmosphere (Jawaid *et al*., 2010). All culture reagents were obtained from Gibco, Lifesciences USA.

Isolation and Manipulation of DNA

Genomic DNA was isolated from MCF7 cells by using QIAamp® DNA Micro kit from Qiagen and visualized on 1% agarose gel.

PCR Amplification of p68 Gene

Oligonucleotides used for amplification were:
sense BglII; 5` GGAAGATCT ACCAT GTCGGGTTATTCGAGTGAC
antisense XhoI: 5` CCGCTCGAG TT AAGCGTAGTCTGGGACGTCGTATGGGTA

Amplification was performed by using GoTaq® Hot Start Polymerase from Promega by following the manufacturer's protocol. Initial denaturation was performed at 94°C for 30 s, 38 cycles (94°C 30 s, 59°C 30 s, 72°C, 1 min 40 s) with final extension at 72°C for 10 min. PCR amplification of the target sequence was confirmed by agarose gel electrophoresis. Digested PCR product was purified by using the QIAquick PCR Purification Kit and digested vector was purified by using QIAquick Gel Extraction Kit from Qiagen.

Cloning of p68 Gene

The ligation of purified PCR p68 sequence was performed by using *T4* DNA *ligase* from Fermantas. DH5α competent cells from Invitrogen were used for transformation of recombinant vectors. Few bacterial colonies obtained after transformation were chosen to select positive clone by plasmid preparation. Colonies were grown overnight in LB medium and used for miniprep analysis by PureYield™ Plasmid Miniprep System (Promega). Ampicillin was used at concentration of 100 mg/mL. Purified recombinant vectors were double digested with respective enzymes from Fermentas by using standard buffering conditions at 37°C for 1 h and 30 min to confirm the presence of insert.

Sequencing and Homology

After restriction analysis positive clones were sequenced from MillGEN, France. The sequence alignment between clone and known *DDX5* sequence was performed by using CLUSTAL W software. BLAST (Basic Local Alignment Search Tool) was used to find similarity in sequence of *DDX5* in different organism. The nucleotide sequence obtained after sequencing corresponding to the gene encoding p68 was used to make Neighbor joining Pylogenetic tree by using MEGA5 software (Yu *et al*., 2012).

Results

PCR Amplification and Cloning of the Gene

MCF7 cells were cultured and maintained in DMEM for isolation of genomic DNA. Isolation of genomic DNA was confirmed by agarose gel electrophoresis. p68 was amplified by PCR using DNA template and amplification was confirmed by agarose gel electrophoresis (Fig. 1). Concentration of the PCR product (ng/µL) was 357.

After PCR amplification; amplified product and vector were double digested with restriction enzymes and purified. After purification p68 was cloned into pMSCV-MIGR1-IRES-GFP vector and confirmed after miniprep analysis. Separation of p68 gene from the vector (Fig. 2) confirmed cloning of the gene.

BLAST (Basic Local Alignment Search Tool) has shown that this partial p68 gene sequence is 99% similar with *Pan troglodytes DDX5* mRNA (AK306198.1), 94% similarity with DEAD box polypeptide 5 (*DDX5*) mRNA (NM_001191395.1) of *Bos taurus* and *DDX5* mRNA (NM_001007613.1) of *Rattus norvegicus* was 93% similar.

Successful cloning produced p68 clone, which can be expressed and purified for further study of its role in regulation of gene expression in different human diseases.

Phylogenetic Analysis

To study the genetic evolution of the human *DDX5*, a phylogenetic tree (Fig. 3) was constructed from the nucleotide sequences retrieved through BLAST (Basic Local Alignment Search Tool). We used neighbor joining method (NJ) to construct the tree. It calculates pairwise distance between sequences and form groups of sequences that are similar. It is computationally simple therefore speedy method (Harrison and Langdale, 2006). Phylogenetic analysis has shown that the deadbox p68 (*DDX5*) of *Human sapiens*, *Sus scrofa* and *Pan troglodytes* are found at equal distance therefore they are evolutionary close. While *DDX5* of *Rattus norvegicus* and *Macaca mulatta* are more divergent from ancestor gene.

It is suggested that *DDX5* of *Human sapiens* and of *Sus scrofa* and *Pan troglodytes* have originated from same ancestors.

Discussion

In present study approximately 2 kb fragment of p68 was cloned from human. Previously, Rössler *et al.* (2000) cloned p68 from human to find its structural organization and found that p68 consisted of 13 exons and 12 introns. Northern blot revealed two forms of p68 mRNA; 2.3 kb fragment corresponding mature mRNA and 4.4 kb fragment representing alternatively or incomplete spliced mRNA.

Fig. 1: PCR amplification of p68 gene from DNA extracted from MCF7 cells. Amplified PCR product was confirmed on 1% agarose gel. Lane 1: 1kb DNA lader, lane 2: blank, lane 3-6 are amplified p68

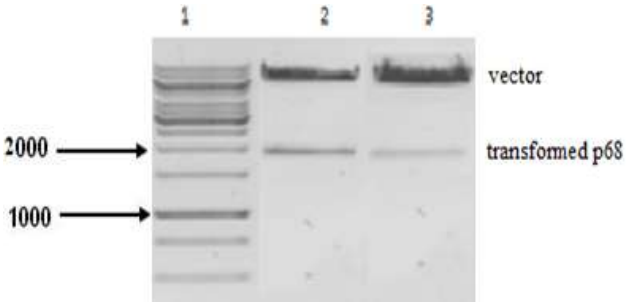

Fig. 2: Restriction analysis of the purified plasmids. Purified plasmids were digested with the same enzymes used for cloning. Digestion products were resolved on 1% agarose gel lane 1, DNA ladder 1kb; lane 2 and 3 are postives clones of p68

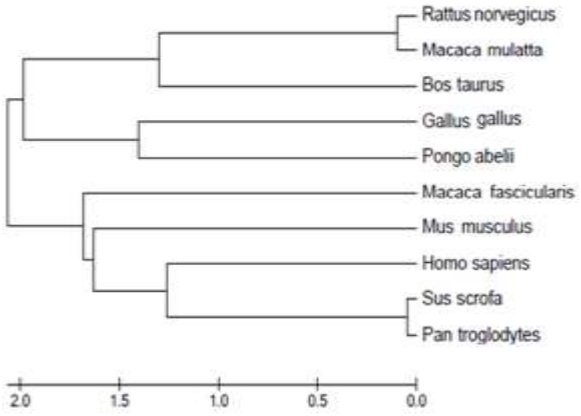

Fig. 3: Phylogenetic tree of *DDX5* gene. A Neighbour Joining (NJ) tree showing the evolutionary relationship among ten different organisms. Branch lengths are proportional to the distance between the sequences

Information that p68 is encoded by a single gene locus in humans came from Iggo *et al*. (1989). Clone prepared during the study can be expressed to study its function in human cells as other researchers have also cloned this gene to study its function. RNA helicases help in transcription either by stabilizing primary transcripts or its release from the template after its completion (Eisen and Lucchesi, 1998). RNA helicase p68 was first identified as 65 kDa protein (p65) by methylene blue-mediated cross-linking (MB-cross-linked) interacting with the U1snRNP when it was bound at 5`ss of intron in spliceosome formation by Liu *et al*. (1998). Later on Liu (2002) used monoclonal antibody PAb204 for immunoprecipitation of HeLa nuclear extract, it precipitated p68. Same antibody also precipitated MB-cross-linked p65. MB-cross-linked and immunoprecipitation with polyclonal antibody PAbN1 (against p68) again precipitated p65 which confirmed that p65 was identical to RNA helicase p68. p68 cross links to 5`ss of RNA component of U1snRNP and is essential for i*n vitro* splicing (Liu *et al*., 1998; Liu, 2002). Alternative splicing of H-Ras is modulated by p68, production of oncogenic p21 H-Ras enhanced in the presence of p68 as it inhibit the inclusion of IDX (Guil *et al*., 2003; Camats *et al*., 2008). Considering this activity, p68 promotes tumor formation by promoting oncogenic p21 H-Ras production. p68 is not only involved in development regulation in humans but in other organisms as well.

The phylogenetic study of this highly conserved p68 encoding gene from *Homo sapiens* has shown its presence in diverse groups of organisms. Both p68 and p72 proteins are involved in cell proliferation/transformation and cellular development in humans (Fuller-Pace, 2006). Recently, Fang *et al*. (2011) identified *DDX5* homolog (Cq-*DDX5*) in *Cherax quadricarinatus* (fresh water crayfish) where this homolog was found to play an important role in early ontogenesis and spermatogenesis.

In conclusion, we have successfully isolated and cloned p68 gene from MCF7 cells. This gene is overexpressed in many of human cancers, therefore, it would be of great interest to overexpress the protein and study how overexpression is involved in the development of cancer? Which level of gene expression regulation is affected by overexpression? The comparison of *DDX5* gene from different organisms would be useful for detailed phylogenetic analysis and to establish its role in those organisms.

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