Localization study of CHD7 protein expressed in HeLa and HEK293 cells

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Abstract. CHARGE syndrome is a rare genetic disease characterized by numerous congenital abnormalities caused by de novo alterations of the CHD7 gene. It encodes a chromo domain protein, involved in the ATP-dependent remodeling of chromatin. It has been described that CHD7 protein plays a role as a positive regulator of the nucleolar expression of the 45S ribosomal RNA precursor. The aim of this research was to study where the CHD7 protein is localized. Knowledge of the subcellular localization of the CHD7 protein is essential for understanding its physiological function. This study was conducted by transfection of two plasmids encoding CHD7-HA or FLAG-CHD7 into HeLa (human cervix carcinoma) and HEK293 (human embryonic kidney) cells. Protein expression and localization of the CHD7 protein was demonstrated by western blot and immunofluorescence. CHD7 protein was expressed in both cell models, and localization of the protein was found in the nucleoplasm, but not in the nucleolus.

1. Introduction

CHARGE syndrome (OMIM 214800) is a rare genetic disease which was described in 1979 when it was recognized independently by two different teams of researchers [1, 2]. This syndrome was initially delineated by Bryan Hall in 17 children with multiple congenital anomalies (MCA) with choanal atresia and, independently, by Hittner in 10 MCA patients with coloboma. In a further review of 21 patients with choanal atresia and/or coloboma, Pagon coined the term ‘CHARGE’, an acronym summarizing six cardinal clinical features: ocular Coloboma, Heart disease, choanal Atresia, Retarded growth and/or development, Genito-urinary defects and/or hypogonadism and Ear anomalies and/or deafness [3]. The prevalence of CHARGE syndrome is ranging from 1/8500 to 1/12000 live births [4, 5].

In 2004, Visser et al. identified molecular abnormalities in patients with CHARGE syndrome using CGH array (Comparative Genomic Hybridization) and FISH (Fluorescent In Situ Hybridization). They concluded that the CHD7 gene is responsible for most cases of CHARGE syndrome [6]. The gene spans 188 kb and consists in 38 exons. Based on previous studies, a mutation in one CHD7 gene allele was found in approximately 2/3 of patients clinically diagnosed with CHARGE syndrome [7, 8]. Therefore, about 35% of patients meeting the clinical criteria of CHARGE syndrome are not carriers of CHD7 gene mutation. Some cases of significant clinical overlap with CHARGE syndrome, such as Kallmann and Omenn-like syndrome have also shown CHD7 mutation [9, 10].
The CHD7 protein belongs to the Chromodomain Helicase DNA-binding (CHD) protein family, which is a highly conserved group of nuclear proteins with nine members in vertebrates. The CHD7 protein consists of 2997 amino acids and belongs to CHD sub-family III characterized by two chromodomains (areas involved in chromatin organization change) in N-terminal position, a SNF2/SWI domain, a helicase domain, a SANT-like domain (Switching-defective protein 3, Adaptor 2 Nuclear receptor corepressor, Transcription factor IIIB) and two C-terminal paired BRK (Brahma and Kismet) domains (figure 1) [11, 12].

The chromodomains are involved in recognition of the ends of lysine-methylated histones, as well as of DNA or RNA targets. The SNF2/SWI is characterized by DNA-dependent ATPase activity involved in the chromatin structure regulation in the eukaryotic genome. Helicase domains play a role in the separation of DNA strands during replication, repair, and recombination. It has been proposed that the SANT domain interaction module permits the coupling between the binding to histone ends and enzymatic catalysis involved in nucleosome remodeling. The function of the BRK domains is unknown, but they are usually found in association with chromodomains [13].

A study conducted by Zentner et al. using DLD1 (human colorectal cancer) cells and a plasmid encoding FLAG-CHD7, with immunodetection method, identified that the location of CHD7 protein expression was predominantly in the nucleoplasm [14]. However, Kita et al. using HeLa and HEK293 cells, using two plasmids encoding EGFP-CHD7 and FLAG-CHD7, identified the location of CHD7 protein in both nucleoplasm and nucleolus [15]. It has been described that CHD7 protein plays a role in various processes, e.g. control and programming of gene expression through ATP-dependent chromatin remodeling process [14]. The CHD7 protein is expressed in early phase of embryogenesis, therefore abnormality in this protein can lead to the various types of congenital anomalies shown in CHARGE syndrome [16]. Several functional studies on CHD7 protein showed that it acts as a transcriptional regulator of precursor 45S rRNA in the nucleolus [14, 17].

Localization of CHD7 protein is necessary for the development of a functional assay for CHD7 protein, particularly in mutated CHD7 gene alleles.

2. Research methods

2.1. Plasmids

Two different plasmids were used to express CHD7 protein. The plasmid that encodes CHD7 tagged with the HA epitope at the C-terminal was provided by Dr. Scacheri (Case Western Reserve University, Cleveland, USA). The plasmid that encodes CHD7 tagged with the FLAG epitope at the N-terminal CHD7 and the plasmid encoding mCherry-nucleolin were given by Dr. Nakayama (Kyushu University, Fukuoka, Japan). The plasmids were purified using the QIAprep Spin Miniprep or Maxiprep Kits (Qiagen, Hilden, Germany) according to the supplier’s instructions. The plasmid concentrations were...
assayed by measuring their absorbance at 260 nm using spectrophotometer BIOMATE 3 (Thermo Scientific, Waltham, Massachusetts, USA).

2.2. Cell culture and transfection
The expression of the CHD7 protein was performed by plasmid transfection into HeLa and HEK293 cells. These cells were cultured under an atmosphere of 5% CO₂ at 37°C in Dulbecco’s Modified Eagles Medium (DMEM, Invitrogen, Carlsbad, California, USA), supplemented with 10% Fetal Bovine Serum (Invitrogen) and 1% antibiotics (Penicillin G and Streptomycin, Invitrogen). For transfections, a 6-well tissue culture plate was used, and the cells were seeded at a cell density of 4x10⁵ cells per well for HeLa and 1.6x10⁶ cells per well for HEK293 in 2 ml of complete culture medium. The cells were incubated overnight at 37°C to obtain 70-80% confluency at transfection. The cells were transfected with the vector using two lipofection (cationic lipid) kits: Lipofectamine 2000 (Invitrogen) and FuGENE6™ (Roche, Basel, Switzerland).

2.3. Cell lysis and protein assay
Forty-eight hours after transfection, the cells were washed three times with PBS to remove the culture medium and dead cells. The cells were lysed using lysis buffer containing 10 mM Tris-HCl pH 7.5, 1% NP-40 and 0.5% DOC and supplemented with 1 mM AEBSF (Sigma-Aldrich, St. Louis, Missouri, USA) and a protease inhibitor cocktail (Roche). Total protein concentration obtained after extraction was measured by the BCA method. Absorbance was measured using spectrophotometer BIOMATE 3 at 562 nm. Then, the next step was denaturation of protein lysates, wherein a mixture of 1 volume of Laemmli buffer (62.5 mM Tris-HCl pH 6.8; 2.3% SDS (v/v), 5% α-monothioglycerol (v/v), 15% glycerol (v/v), 0.001% bromophenol blue (v/v)) and 3 volume of lysate was incubated at 95°C for 5 minutes.

2.4. Western blot
The samples were separated on a 5% SDS-polyacrylamide gel (SDS-PAGE). The migration of the proteins was carried out at a current of 34 mA in SDS-PAGE buffer (SDS-10TGX, Amresco). The progress of the migration can be followed by a colored mass marker (Kaleidoscope™, Bio-Rad, Hercules, California, USA). Thereafter, the separated proteins were transferred from the gel onto a Hybond ECL nitrocellulose membrane (GE Healthcare, Chicago, Illinois, USA) in the presence of transfer buffer (ethanol 1 L; TG SDS-10X 250 mL; H₂O 3.75 L). The primary antibody diluted in PBS Tween supplemented with 5% skimmed milk was applied to the membrane at 4°C with stirring overnight. After three additional washings in 0.1% PBS-Tween, the membrane was incubated with secondary antibody coupled to peroxidase, diluted in PBS-Tween supplemented with 5% skim milk for one hour. The bindings of antibody to the membranes were recognized using chemiluminescence detection system ECL Plus Western Blotting Detection System (GE Healthcare) and the proteins of interest were detected by exposure to photographic films Hyperfilm ECL (GE Healthcare).

2.5. Immunofluorescence
The cells were cultured on a glass coverslip. The cells were incubated overnight with 30 µl primary antibody diluted 1/100 in 0.5% PBS-BSA at 4°C in a humid chamber. The next day, the slides were washed three times in PBS-BSA and incubated 1 hour in the presence of 30 µl of secondary antibody (diluted 1/100 in PBS-BSA) supplemented with DRAQ5™ (eBioscience, Waltham, Massachusetts, USA) to stain the nucleus. The coverslips were mounted on slides with mounting medium (Moviol®, Sigma-Aldrich). Immunofluorescence localization of the proteins was visualized using a confocal microscope Olympus FV1000 (Shinjuku, Tokyo, Japan).

2.6. Antibodies
Anti-UBF was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA); anti-HA, anti-FLAG, and anti-actin were from Sigma-Aldrich. Horseradish peroxidase-coupled anti-mouse and anti-
rabbit IgG antibodies were from Sigma-Aldrich. Fluorescent anti-mouse and anti-rabbit IgG antibodies were from Jackson ImmunoResearch (Cambridgeshire, United Kingdom).

3. Results and discussion

3.1. Immunoblot of CHD7 protein
In the initial experiments, we established optimal transfection conditions in 6-well dishes. Plasmids encoding CHD7-HA or FLAG-CHD7 were transiently transfected into HeLa and HEK293 cells. As a negative control, an expression vector that does not contain the desired gene (mock) was used. On the one hand, we tested a range of 1, 2, 4 and 8 µg plasmid with 5 or 10 µL of Lipofectamine reagent per well; on the other hand, we tried 1 or 2 µg of the plasmid with 3 µL of FuGENE per well. Two different exposure times of cells to the complexes were applied: 48 hours and 72 hours.

The transfection efficiency was assessed by western blot, using anti-HA or anti-FLAG primary antibody to test CHD7 protein expression (expected molecular weight of 340 kDa). In parallel, the detection of β-actin protein was also performed to verify the quality of the protein lysate and the uniformity of the protein amount loaded into each well. The highest transfection efficiency in HeLa and HEK293 cells was obtained within 48 hours by using 4 µg of plasmid DNA with 10 µL of Lipofectamine per well (figure 2).

![Figure 2](image)

**Figure 2.** FLAG-CHD7 protein expression. HeLa (A) and HEK293 cells (B) were transfected with an empty vector (mock) or with a plasmid encoding FLAG-CHD7. 100 µg (resp. 30 µg) of protein lysates were separated by SDS-PAGE and analyzed by immunoblotting with an anti-FLAG antibody (resp. anti-actin as a loading control). CHD7-HA was also evidenced by western blot in both cell models (not shown).

On the contrary, the transfection using FuGENE was unsuccessful, although it had been repeated in various concentrations of plasmids. In conclusion, the transfection of cultured HeLa and HEK293 cells is effective only when using Lipofectamine reagent.

3.2. Localization of CHD7 protein
To investigate the subcellular localization of the protein, HA-tagged CHD7 was expressed in HeLa and HEK293 cells. Double immunostaining was performed against CHD7-HA and endogenous UBF, a protein expressed in the nucleoplasm and highly concentrated in the nucleolus (figure 3).
Figure 3. Localization of CHD7-HA protein. The CHD7-HA was expressed in HeLa (top) and HEK293 cells (bottom). Immunostaining was visualized using confocal microscopy, the CHD7 protein was detected in green and UBF protein in red. The DRAQ5 dye was used to color the nuclei (blue). CHD7-HA protein was identified only in the nucleoplasm. Bar: 10 µm.

We observed that CHD7-HA was present throughout the nucleoplasm but absent of the nucleolus. Therefore, we performed in both cells a co-transfection experiment, to express FLAG-tagged CHD7 and a fusion protein between nucleolin (localized exclusively in the nucleolus) and the red fluorescent protein mCherry (figure 4). Contrary to the literature, repeated experiments performed the same results showing that CHD7 protein localizes exclusively in the nucleoplasm, but not in the nucleolus.

Figure 4. Localization of FLAG-CHD7 protein. HeLa (top) and HEK293 cells (bottom) were co-transfected to express FLAG-CHD7 and mCherry-nucleolin. The fluorescence was visualized using confocal microscopy. FLAG-CHD7 protein was detected in green and nucleolin in red. DRAQ5 dye was used to color the nuclei (blue). FLAG-CHD7 protein was localized only in the nucleoplasm. Bar: 10 µm.

In this study, the early stage of our experiments permitted to determine the optimal transfection conditions to overexpress CHD7 protein. We obtained the highest transfection efficiency in both cell models using Lipofectamine reagent, contrary to the transfection using FuGENE, which was not achieved although it had been repeated with various concentrations of plasmids. The reasons for the
failure of transfection using FuGENE remain unclear, but it most likely depends on cell type specificity and on which protein is to be overexpressed.

When we analyzed protein expression using western blot, a weak signal was found. This can be explained as follows. First, the experimental conditions are not optimal for western blotting: the quite large size of the CHD7 protein (340 kD) is allegedly one of the causes of weak signal. Second, low transfection efficiency will lead to a low level of protein expression as well. This has been confirmed in immunofluorescence experiments, in which not all cells express CHD7, in both cell lines. These difficulties may also be caused by the large size of the recombinant plasmid (approximately 16 kb).

Localization of CHD7 protein was determined by immunostaining. Previous studies showed that CHD7 is predominantly localized in the nucleolus [14, 15]. In our study, we expressed in HeLa and HEK239 cells two forms of CHD7 protein, tagged in N- or C-terminus, and we used two distinct nucleolar markers, but we did not observe any nucleolar localization for CHD7 protein. It is still unclear why CHD7 was always localized exclusively in the nucleoplasm and not in the nucleolus. It may be due to disruption in transport to nucleoli of newly synthesized CHD7 protein.

Nevertheless, it may be more appropriate to carry out this study in cellular models in which CHD7 is endogenously expressed: for example, CHD7 is selectively expressed in neuronal stem cells (NSC) and neuronal progenitor cells [18]. In addition, to verify and confirm the localization of expressed CHD7 protein, western blot analysis of separated subcellular fractions (cytoplasmic, nucleoplasmic and nucleolar proteins) could be performed [16]. Moreover, N- or C-terminus fusion tags may interfere with the addressing of CHD7. Therefore, it may be more appropriate to express the CHD7 protein using a plasmid encoding untagged CHD7 and perform immunostaining with an anti-CHD7 antibody.

4. Conclusion
Localization of CHD7 protein was determined by immunostaining. CHD7 protein was expressed in HeLa (human cervix carcinoma) and HEK293 (human embryonic kidney) cells, and localization of the protein was found in the nucleoplasm, but not in the nucleolus.

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