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Polyhistidine tract expansions in HOXA1 result in intranuclear aggregation and increased cell death

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Abstract

HOXA1 gene is part of a cluster of homeotic selector genes that regulates the anteroposterior patterning of mammals during embryonic development. HOXA1 encodes two alternatively spliced mRNAs with two isoforms, A and B, the former contains the homeodomain and expressed in early embryonic development. HOXA1 contains a string of 10 histidine repeats. However, individuals heterozygous for 7, 9, 11, and 12 histidine repeat variants were present among the Japanese population, notably in some autism cases. To determine the biological implications of the different polyhistidine repeat lengths, we expressed these variants in COS-7 and a human neuroblastoma cell line (SK-N-SH). Expression of expanded variants of HOXA1 isoform A, containing 11 and 12 polyhistidine, resulted in early and great degree of protein aggregation in the nucleus. This aggregation resulted in accelerated cell death in cells expressing 11 and 12 expanded variants compared to those transfected with 7 and 10 polyhistidine variants. Furthermore, we showed that these aggregates were ubiquitinated and were inhibited by a histidine-modifying compound, DEPC. These data suggest that HOXA1 protein with polyhistidine tract expansions misfold, aggregate, and have a toxic effect on cell.

Keywords: HOXA1; Histidine repeat; Protein aggregation; Ubiquitin; Cell death; Diethylpyrocarbonate

Homeobox genes encode for transcription factors contributing to the regulation of embryonic patterning and organogenesis [1]. The clustered homeobox genes were highly conserved from *Drosophila* to human at the genomic level. In mammals, 39 of the Hox genes were identified and they encode to the class of proteins which share the evolutionary conserved homeodomain involved in the recognition of the target DNA sites [2]. HOXA1 is the first homeobox gene expressed in the developing hindbrain [3]. Its mRNA has two alternative splice variants; one containing the homeobox which encodes isoform A, and the shorter, isoform B, lacking the homeobox [4]. One of the noticeable features of HOXA1 isoform A is a stretch of 10 histidine repeats at amino acid positions 65–74. Although, its importance has not yet been established, it may possibly interact with Polycomb, a repressor of homeotic genes. The targeted disruption of the Hoxa1 gene in mice leads to numerous developmental defects, including hindbrain deficiencies and abnormal skull ossification and ultimately to neonatal death [5,6]. Ingram et al. [7] reported

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an association of the A218G polymorphism in HOXA1 gene and autism; however, a number of contradictory reports have also been made [8,9]. Racial differences of sample populations may be the reasons for these discrepancies.

In this study, we investigated HOXA1 gene variations in a Japanese population comprised of normal and autistic individuals, and found novel histidine repeat deletions and expansions. Expression of polyhistidine expanded forms of HOXA1 protein results in early intranuclear aggregation and increased cell death. Time-dependent protein aggregation and cell death could be an indication of apoptosis associated with the process of assigning segmental identity in the developing hindbrain. Moreover, expanded forms of HOXA1 isoform A, aggregated earlier and faster, suggest that longer histidine repeats may result in accelerated apoptosis.

Materials and methods

Antibodies and reagents. Antibodies were purchased as follows, polyclonal anti-HOXA1 antibody (N-20) was from Santa Cruz, polyclonal anti-EGFP antibody was from Invitrogen, and polyclonal anti-ubiquitin antibody, Alexa fluor 546-conjugated anti-rabbit IgG antibody, and Alexa fluor 594-conjugated anti-goat IgG antibody were from Molecular Probes. Hoechst 33258 for nuclear staining was from Molecular Probes.

DNA sequencing of HOXA1 gene. Subjects consisted of 110 Japanese autistic patients, their parents ($n = 211$); and 336 normal controls. All autistic patients were diagnosed under DSM-IV criteria. This study was approved by the Ethical Committees of the Faculty of Medicine, University of Tokyo, and the Faculty of Medicine, Tottori University. Total DNA was extracted from lymphoblasts using a standard method. PCR primers were designed to amplify all exons of HOXA1, including flanking sequences. PCR products were subcloned in T-Vector (Promega) and sequenced.

HOXA1 plasmid constructions. Each HOXA1 isoform was amplified from lymphocyte mRNA of human normal controls and autistic individuals following the standard method. Since isoform A differs from isoform B primarily by the presence of the homeodomain region and expresses only in the developing embryo, isoform A constructs were generated partly from genomic DNA by PCR amplification and joined with the appropriate region from isoform B transcript. All PCR amplification steps were performed using Pfu Ultra DNA polymerase (Stratagene) and confirmed by sequencing. Each variant was ligated into pCMV-Script (Stratagene) and pEGFP-N1 (Invitrogen) to generate expression constructs.

Transfection and cell death assay. COS-7 and human neuroblastoma cell line (SK-N-SH) were maintained in DMEM (Sigma) with 10% fetal bovine serum (Gibco) and transfected with HOXA1 constructs using Fugene 6 transfection reagent (Roche) following manufacturer's protocol. After transfection, cells were maintained in DMEM supplemented with 10% serum. A mock transfection was also performed as a control. Floating dead cells from medium of transfected cells were harvested every 24 h for a period of 3 days, stained with Trypan blue (Gibco), and counted using a hemocytometer under a phase contrast microscope (Olympus IX-70). Lactate dehydrogenase (LDH) cytotoxicity assay (Wako, Tokyo) was also performed from the collected cell medium following the manufacturer's procedure.

Immunocytochemistry and fluorescence imaging. After transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and immunostained with anti-HOXA1 antibody and anti-ubiquitin antibody for 1 h at room temperature. Bounded antibodies were detected by incubation with Alexa fluor 595-conjugated anti goat IgG antibody for 1 h. For nuclear staining, cells were incubated with Hoechst 33258 for 30 min. All samples were mounted on glass slides and fluorescence images were obtained using Leica confocal microscope (TCS SP2).

Western blotting. Twenty-four hours after transfection, cells were harvested for total protein extraction. Briefly, cell samples were lysed and sonicated with 10 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA plus protease inhibitor cocktail (Roche). Protein was quantified using Color-Producing Solution (Wako). Samples were run on 10% SDS–PAGE gels and transferred on a nylon membrane (Millipore) using a semi-dry transfer blotter (Bio-Rad). Membranes were incubated in either a polyclonal rabbit anti-EGFP antibody followed by incubation in a horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham), respectively, or in a polyclonal anti-HOXA1 antibody with a horseradish peroxidase linked sheep anti-goat antibody (Amersham). Detection was performed using ECL (Amersham–Pharmacia Biotech) and images were captured in X-ray film or a LAS-1000 plus imager (Fujifilm).

DEPC treatment. Twenty-four hours after transfection, cells were treated with diethylpyrocarbonate (DEPC) with a concentration of 20 mM for 30 min or 1 mM for 24 h. Then cells were fixed and mounted on the slides. The degree of protein accumulation within the cell nuclei of the transfected cells was counted. Cells were counted from 10 randomly selected microscope fields from each sample. The ratio of the number of aggregated cells over the total number of EGFP-positive cells was then computed. The same computation procedures were performed for cells processed after 18 and 42 h of incubation.

Results

Novel variants of histidine repeats in HOXA1 gene

All exons and flanking sequences of HOXA1 gene were subjected to direct sequencing. Using this approach, novel polyhistidine variants were detected. Families affected with autism and randomly selected normal individuals were screened for sequence of HOXA1 gene. Finally 7, 9, 11, and 12 repeat variants were detected in both normal and autism patients (Table 1). Nevertheless, we were interested in further expression study because some trinucleotide repeat expansions, such as polyglutamine and polyalanine, have already been implicated neurodegenerative and congenital diseases [10–12].

Intranuclear protein aggregations in polyhistidine variants of HOXA1

To investigate the cellular localization of the different variants of HOXA1, we generated expression constructs having the 7, 10, 11, and 12 polyhistidine repeats for the

Table 1
Allele frequencies of the polyhistidine variants in HOXAI gene between autism patients (child), their parents, and normal individuals

Type	Child	Parent	Normal
7/10	2	4	11
9/10	0	0	1
10/10	105	200	312
11/10	3	6	12
12/10	0	1	0
Total	110	211	336

The length of histidine repeats in HOXAI gene was assessed by sequencing of genomic DNA and was classified under five types of allelic variants.