Down-regulation of heat shock protein 27 in neuronal cells and non-neuronal cells expressing mutant ataxin-3

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Abstract Machado–Joseph disease (MJD)/spinocerebellar ataxia type 3 is an autosomal dominant spinocerebellar degeneration characterized by a wide range of clinical manifestations. Unstable CAG trinucleotide repeat expansion in the MJD gene has been identified as the pathologic mutation of MJD. In this study, human SK-N-SH neuroblastoma cells stably transfected with full-length MJD with 78 CAG repeats were established. Compared with the parental cells, cells expressing mutant ataxin-3 displayed normal morphology for over 80 generations. Less than 1% of the transfected cells contained nuclear aggregates under basal conditions, indicating that this cellular model represented an early disease stage. While *t*-butyl hydroperoxide (TBH) was used to assess the oxidative tolerance of cells, the results demonstrated that the transfected cells were more susceptible to low concentrations of TBH than the parental cells. Most interestingly, from 2D gel electrophoresis analysis, we identified that the expression of heat shock protein 27 (HSP27), known as a suppressor of poly(Q)-mediated cell death, dramatically decreased in SK-N-SH cells stably transfected with full-length mutant MJD. The same reduction of HSP27 was further confirmed in lymphoblastoid cells from MJD patients. Our results demonstrated that both neuronal and non-neuronal cells with expanded full-length ataxin-3 revealed reduced protein expression of HSP27. We propose that the reduction of HSP27 in the early stage of the disease plays an important role during cell death process in MJD.

Key words: Machado–Joseph disease; Full-length mutant ataxin-3; Oxidative stress; Heat shock protein 27

1. Introduction

Machado–Joseph disease (MJD) belongs to a special class of inherited neurodegenerative disease caused by CAG trinucleotide repeat expansion in the coding region of the respective genes [1,2]. In all cases, the CAG repeats are transcribed and translated into polyglutamine tracts [3]. Clinically, MJD is characterized by progressive ataxia in combination with various non-cerebellar symptoms, including oculomotor abnormalities, spasticity, basal ganglia symptoms, peripheral neuropathy and cognitive disturbances [4,5]. All MJD-affected patients exhibit expanded CAG's with 55–84 repeats whereas normal individuals exhibit 13–51 repeats [6]. Polyglutamine diseases are dominantly inherited, typically late-onset, fatal neurodegenerative disorders. The protein is widely expressed in neurons [7] and outside the central nervous system, but the mutation ultimately leads to selective neuronal loss in restricted brain regions. The nature of the toxic insult of a poly(Q) mutation and its biological consequences in each disease are unclear. It is possible that the poly(Q) expansion interferes with basic cellular process such as transcription, protein degradation and survival/death signaling [8]. It was shown that the ataxin-3 accumulated in ubiquitinated intranuclear inclusions selectively in neurons of affected brain regions [9]. Neuronal intranuclear inclusions have become the neuropathological sign of the CAG repeat diseases, but their cytotoxicity still remained controversy [10].

The mechanism that leads the polyglutamine-expanded proteins to aggregate is unknown. There is a possibility that the extended polyglutamine tract may destabilize the protein to misfold and aggregate. Indeed, in spinocerebellar ataxia type 3 (SCA3) brain, heat shock protein 40 (HSP40) and HSP70 were found to localize to nuclear inclusions (NIs) [11]. In addition, different HSPs have been shown to directly inhibit several types of cell death pathways induced by a variety of toxic insults in neuronal cells [12–15]. HSP27 was reported to have anti-apoptotic properties in neuronal survival [16,17]. The expression of HSP27 was shown to enhance the survival of mammalian cells exposed to a number of cytotoxic agents, including heat shock, oxidative stress, chemotherapeutic agents, and cytokines [18–21]. In addition, oxidative stress induced by reactive oxygen species (ROS) or free radicals played an important role in the pathogenesis of several neu-

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rodegenerative disorders including Alzheimer's disease [22], Huntington disease and other late-onset neurodegenerative conditions [23]. Recently, it was reported that overexpression of HSP27 prevented cellular polyglutamine toxicity and suppressed the increase levels of cellular ROS caused by huntingtin [24]. However, the mechanisms of how HSP27 involved in the pathogenesis of MJD remained unclear.

In cells transiently transfected with expanded ataxin-3 and in the human SCA3 brain tissue, transcription factors CBP (cAMP response element-binding protein-binding protein) and TBP (TATA-binding protein) were recruited into NIs, pointing to a direct interaction of the expanded ataxin-3 with specific transcription factors [25,26]. In addition, rate mesencephalic CSM14.1 cells stably expressing expanded ataxin-3 resulted in the up-regulation of some inflammatory genes in the late stage of the disease [27]. However, to our best knowledge, no human neuronal cells expressing full-length mutant ataxin-3 were previously reported. In the present study, we established SK-N-SH neuroblastoma cells stably expressing anti-hemagglutinin (HA)-tagged full-length MJD with 78 CAG repeats to examine the effects of expanded ataxin-3 under normal conditions and oxidative stress. Our results showed that only less than 1% of the mutant cells contained nuclear aggregates under basal growth conditions. Stably transfected cells were more sensitive to low concentrations of *t*-butyl hydroperoxide (TBH), indicating that transfected cells were more susceptible to the toxic insult than cells without mutant ataxin-3. Most interestingly, we demonstrated that the protein levels of HSP27 dramatically decreased in cells with expanded ataxin-3, which may significantly impair the protection ability of the cells to respond to stress and ultimately lead to stress-induced cell death.

2. Materials and methods

2.1. Materials

SK-N-SH cells were provided by Dr. Shin-Lan Hsu (Taichung Veterans General Hospital, Taiwan). All culturing supplies and transfection materials were obtained from Gibco Life Technologies (Gaithersburg, MD, USA). Western blot reagents were obtained from Pierce (Rockford, IL, USA). Mouse monoclonal anti-MJD was made previously [28]. Mouse monoclonal anti-HSP27 was from Lab Vision (Fremont, CA, USA). Monoclonal anti-β-actin was from Sigma (St. Louis, MO, USA). Mouse monoclonal HA epitope was from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit polyclonal HA epitope was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other supplies were obtained from Sigma Chemical (St. Louis, MO, USA).

2.2. Generation of stably transfected MJD cells

Neuronal SK-N-SH cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. Cells in 60-mm dishes, at 60–70% confluence, were transfected using Lipofectamine reagent following manufacturer's instructions. Cells were transfected with 2 μg of empty pCDNA3.1 vector or pCDNA3.1 containing full-length MJD with 78 glutamines (pCDNA3-HAMJD78, a generous gift from Dr. Henry Paulson). Twenty-four hours after transfection, cells were selected in culture medium supplemented with G418 (neomycin sulfate, 500 μg/ml). Densely growing foci of transformed cells were trypsinized after 2 weeks and transferred to a 6-well plastic culture dish. Transfection with the empty plasmid was also performed as comparison. Selected G418-resistant cells were subcloned and maintained in the same conditioned medium until each cell line contained homogenous transfected cells. After 3 months of selection and subcloning, we obtained stable cells expressing expanded ataxin-3. For all experiments, cells were placed in medium lacking G418.

2.3. Preparation of lymphoblastoid cells

Lymphoblastoid cells from two MJD-affected patients with alleles containing 31 and 80, 16 and 79 CAG repeats, respectively, and normal control with 12 CAG repeats were established by Epstein–Barr virus transformation. The procedure details were as described [29,30]. Cells were maintained in RPMI 1640 medium (Gibco-BRL) containing 10% FBS.

2.4. Immunocytochemical staining

For immunocytochemical staining, SK-N-SH or SK-N-SH-MJD78 cells were fixed in 4% paraformaldehyde for 20 min, following by three washes in phosphate-buffered saline (PBS), 20 min incubation with 1% Triton X-100 in PBS to permeabilize the cells, 20 min incubation with 2% bovine serum albumin in PBS to block non-specific binding, and then incubation with each antibody at 4°C overnight. After washing with PBS, immunodetection was performed with biotinylated anti-mouse immunoglobulin followed by peroxidase-labeled streptavidin (BioGenex). Finally, the reactants were developed with 3,3′-diaminobenzidine (Dako). Counterstaining was performed using hematoxylin. Visible aggregate formation was determined using a Zeiss fluorescence microscope equipped with a digital camera. The percentage of cells exhibiting aggregates was calculated. In each experiment at least 30 cells were counted per dish, with a minimum of four dishes utilized for each time point.

2.5. Cell toxicity studies and analysis of cell survival

SK-N-SH and SK-N-SH-MJD78 cells were maintained in DMEM medium containing 10% FBS and then were prepared at a concentration of 1×10^5 cells/ml, 100 μl of which was added to each well of 96-well plates. One day after seeding, cells were changed to medium containing no serum for 24 h and then TBH (0 μM, 1 μM or 3 μM) was added to the cells. Appropriate controls were included with only water added to cells. After 12, 24, 36, 48, 60 h, cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay (cell titer 96, Promega) as described previously [31,32]. At least six cultures were utilized for each time point. After 4 h of incubation, optical densities were measured at 490 nm. Results were expressed as the percentage of controls.

2.6. Western blot analysis of protein levels

In brief, cell lysates containing 20–30 μg of protein were loaded onto 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels. Resolved proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking the membrane with 5% non-fat milk in NaCl/Pi/0.1% Tween 20 for 1 h at room temperature, all antibody-binding reactions were performed in the same buffer supplement with 1% non-fat milk at 4 °C overnight for primary antibodies and at room temperature for 1 h for secondary antibodies coupled to horseradish peroxides. The signal was detected by the enhanced chemiluminescence supersignal system (Pierce, Rockford, IL, USA). Monoclonal antibody against β-actin was included in the experiments as an internal control.

2.7. Two-dimensional gel electrophoresis

We applied 500 μg of each cell lysate at the cathodic end of the immobilized pH gradient gel (IPG) strips (pH 3–10, 13 cm, Pharmacia Biotech, Uppsala, Sweden), and isoelectrophocusing (IEF) was conducted for 10–16 h (13 000–20 100 V/h) using Electrophoresis Power Supply ESP 3500 XL (Pharmacia Biotech). Upon completion of IEF, the IPG strips were equilibrated in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 15.4 mg/ml dithiothreitol, and 0.0125% bromophenol blue for 15 min. The strips were stored at 80°C or used immediately. Gradient SDS–polyacrylamide gels (5–20%, 17 cm×20 cm×1 mm) were employed for the two-dimensional separation in a PROTEAN II xi 2D cell (Bio-Rad, Hercules, CA, USA), and run at 40 mA constant current for 5 h. The two-dimensional gels were stained with Coomassie brilliant blue G-colloidal. Destaining was performed by soaking the gels in 10% acetic acid and 25% methanol solution for 60 s, then in 25% methanol solution for 24 h at room temperature. The two-dimensional maps of cells were compared by using Z3 (Compugen) or Melanie III 2D polyacrylamide gel electrophoresis software (Genebio, Geneva, Switzerland) and checked manually. Proteins were recovered by punching out spots with Pipet Tips (volume: 100–1000 μl). Protein spots were punched for later mass spectrometry analysis.

2.8. In-gel enzymatic digestion

The gel pieces were washed twice with 30 μl of 25 mM ammonium bicarbonate/acetonitrile (1/1 v/v, pH 8.5) and followed by dehydration with acetonitrile for several times. After complete drying in a speed vac for 15 min, gel pieces were placed overnight at 37°C in a digestion buffer containing 50 ng/μl sequencing grade trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate. The obtained peptides were extracted with 10 μl of water–acetonitrile (1/1 v/v, 0.5% trifluoroacetic acid (TFA)) for three times. The combined fractions were lyophilized to dryness. The peptides were further desalted with Ziptip (Millipore, Bedford, MA, USA) and dissolved in 10 μl of water–acetonitrile (1/3 v/v, 0.1% TFA).

2.9. Tandem electrospray ionization mass spectrometry (ESI-MS/MS)

MS/MS sequence of peptides generated by in-gel digestion was performed by high-performance liquid chromatography-coupled nano-ESI on a Q-TOF II mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray source. The peptides were separated on a 150-mm id/10-mm length C18 RP column. Elution of peptides was carried out with a gradient solution of 90% acetonitrile over 25 min at a flow rate of 200 nl/min. The eluted peptides were analyzed by sequencing with automated MS-to-MS/MS switching protocol. MS acquisitions were performed within the mass range of 400–2000 *m/z* and MS/MS within 50–3500 *m/z*. The spectral data were processed using MassLynx 3.4 software to obtain centroid MS/MS data. The resulting PKL files were submitted to the MASCOT (http://www.matrixscience.com) search engine that was set to query NCBI database to obtain the corresponding protein identity.

2.10. Statistical analysis

All bands were quantified by laser densitometry. All values were expressed as means ± S.E.M. The analysis of variance with subsequent Student's *t*-test was employed to determine the significance of differences in comparisons. Values of $P < 0.05$ were considered statistically significant.

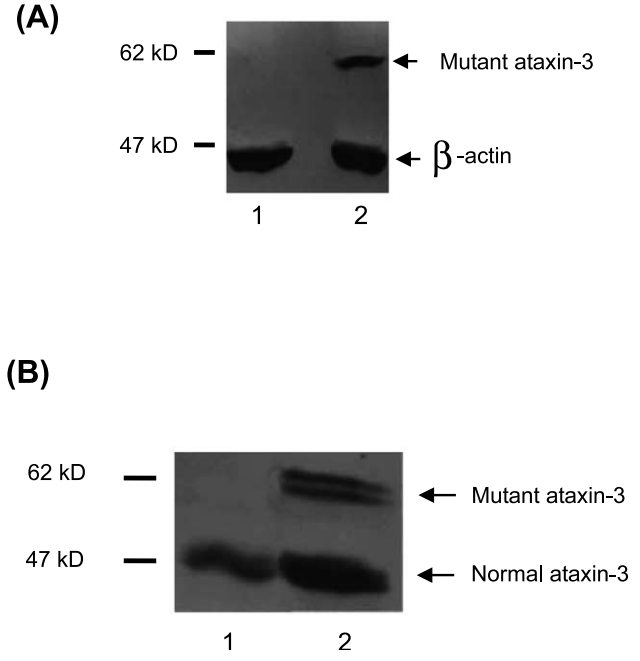


Fig. 1. Detection of expressed wild-type and expanded ataxin-3. A: Western blot analysis of the parental SK-N-SH cells (lane 1) and stably transfected SK-N-SH-MJD78 (lane 2). Western blot probed with anti-HA and anti-β-actin antibodies. B: Western blot analysis of the parental SK-N-SH cells (lane 1) and stably transfected SK-N-SH-MJD78 (lane 2). Monoclonal anti-ataxin-3 was used in the blotting.

3. Results

3.1. Expression of expanded ataxin-3 in SK-N-SH cells

Although an inducible rat mesencephalic CSM14.1 clonal cell line expressing expanded human full-length ataxin-3 was previously reported [27], a human neuronal cell line expressing mutant ataxin-3 would be valuable to study the effect of mutant ataxin-3. Therefore, human SK-N-SH cells, containing endogenous normal ataxin-3 with 26 glutamine residues, were used as the parental cells. SK-N-SH cells were stably transfected with full-length poly(Q) expanded MJD construct (pCDNA3-HAMJD78), using G418 (500 μg/ml) to select stably transfected cells. A stable clone, named SK-N-SH-MJD78, was obtained after 3 months selection. Expression of the exogenous mutant ataxin-3 in SK-N-SH-MJD78 was confirmed by Western blot analysis and immunocytochemistry. Antibodies against ataxin-3 or HA epitope were used for blotting because the pCDNA3-HAMJD78 constructs were HA-tagged. A representative Western blot is shown in Fig. 1A. A band of ~62 kDa was visible on a 6% SDS–polyacrylamide gel in the transfected cells (lane 2) representing the expression of HA-tagged expanded ataxin-3, whereas the parental SK-N-SH cells did not show the signal (lane 1). In addition, when immunoblotted by monoclonal antibody to ataxin-3, the results showed that both normal and expanded (26 and 78 residues) ataxin-3 could be visible in the transfected cells (Fig. 1B, lane 2) compared with only one normal signal (26 residues) in parental cells (Fig. 1B, lane 1). It was noted that another immunoreactive protein was also seen as a doublet at the size of 62 kDa corresponding to the length of the CAG expansion. The latter was also probed with 1C2 (data not shown). The extra band of expanded ataxin-3 may indicate alternatively spliced transcripts existing or the cleavage of HA-tag in the stably transfected neuronal cells.

From immunocytochemical study, full-length ataxin-3 with expanded repeat (78 residues) usually diffusely distributed in the cytoplasm of transfected cells (Fig. 2B), compared with very little background staining in the control cells (Fig. 2A). Less than 1% of the transfected cells showed NIs (Fig. 2C) and both SK-N-SH and SK-N-SH-MJD78 showed similar morphology. Size and stability of the exogenously expressed ataxin-3 was further confirmed on lysates obtained from cells at the 11th, 30th and 80th passages (data not shown). The intensity of the immunoreactive band in cells bearing the mutant ataxin-3 did not significantly alter with time in vitro, up to the 80th passage. In addition, the size of the immunoreactive band remained the same, indicating that the poly(Q) repeat was stably replicated in SK-N-SH-MJD78 cells. To date, SK-N-SH-MJD78 cells have been passaged for over 80 generations under basal conditions without apparent loss of phenotypes. The transfected cells before the 60th passage were used for the following experiments.

3.2. Wild-type and mutant MJD differentially affect cell viability upon TBH treatment

Due to the late-onset feature of the disease, we hypothesized that oxidative stress might be contributory to the progression of the disease. To test this hypothesis, the oxidative stress derived from exogenous sources was evaluated in our cellular model. TBH, widely used as a mild oxidative reagent in different cellular models [33], was used to assess the oxidative tolerance of cells carting mutant ataxin-3. While neuronal

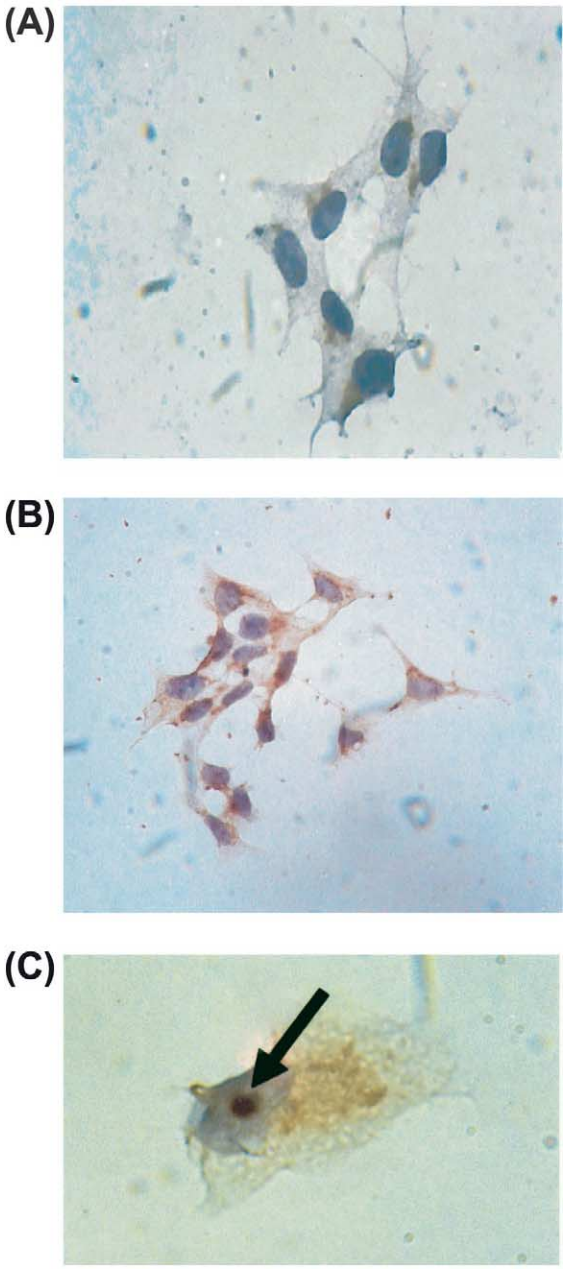


Fig. 2. Immunocytochemical analysis of expressed wild-type and expanded ataxin-3. A: SK-N-SH (magnification: 1000×). B: SK-N-SH-MJD78 cells were labeled with monoclonal anti-HA antibody (magnification: 400×). C: About 1% of the stably transfected cells showed NIs with monoclonal anti-ataxin-3 antibody. The arrow indicates the NI body (magnification: 1200×).

cells were exposed in vitro to TBH from 1 to 3 μM, quantitations of the effects by MTS assays revealed significant differences in cell viability between cells with (Fig. 3A) and without (Fig. 3B) expanded ataxin-3. Fig. 4B shows that cultures of the parental SK-N-SH cells did not exhibit a dose-dependent decrease of cell viability after 1 and 3 μM TBH treatment. However, this decrease was evident in SK-N-SH-MJD78 cells (Fig. 3A). It was noted that increased sensitivity to TBH up to 24 h treatment in the transgenic cell line compared to control cells. The 36 and 48 h values rather indicated some kind of proliferation phenomenon of both controls and transgenic cells. It is possible that TBH was not stable after 24 h treatment or some intrinsic antioxidative protection properties might have been induced.

3.3. Protein expression of HSP27 dramatically decreased in the presence of expanded ataxin-3

Meanwhile, because the low percentage of NIs was observed in SK-N-SH-MJD78, our cellular model was valuable to study the early events of MJD. Comparative proteome analysis was performed to distinguish the putative gene expression alterations between cells with and without expanded ataxin-3. One candidate spot with a significant expression difference (Fig. 4A) was excised, trypsin-digested and analyzed by ESI-MS/MS. Mass spectra corresponding to tryptic digest of this protein are shown in Fig. 4B. The MS/MS data was matched to HSP27 in NCBI database using the MASCOT search engine (http://www.matrixscience.com). The amino sequence of HSP27 is shown in Fig. 4B and the essential peptides used for MS/MS sequencing were underlined. MS/MS profile of the investigated peptide 582.24 (2+) clearly indicated the amino acid sequence as *LFDQAFGLPR*, which is characteristic to HSP27 (Fig. 4C). In order to confirm this observation, Western blot analysis was performed using monoclonal

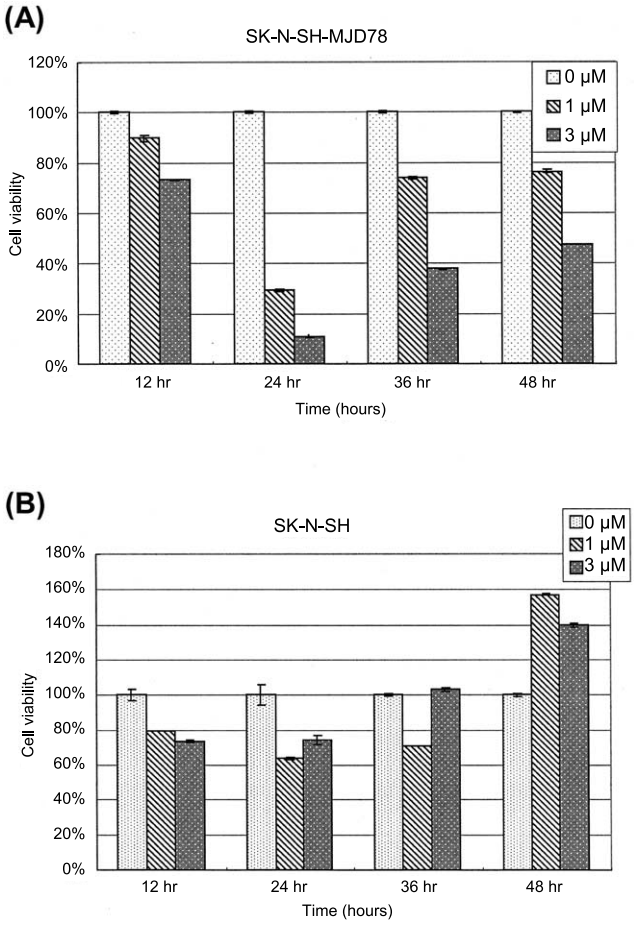
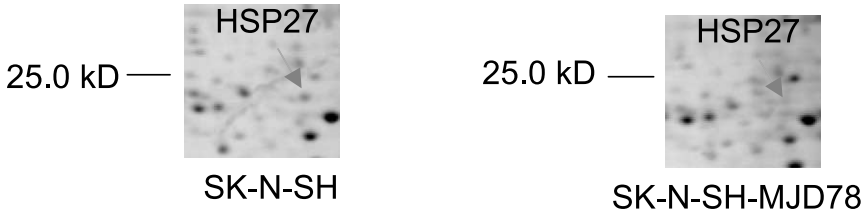


Fig. 3. SK-N-SH-MJD78 cells are more susceptible to oxidative stress by TBH. Characteristics of cell death induced by expanded ataxin-3 under TBH treatment. SK-N-SH-MJD78 (A) and SK-N-SH cells (B) were treated with TBH (1 or 3 μM) for various times at 37°C. Cell viability was estimated by the MTS assay. The values were normalized to 100% using the respective control cells not treated with TBH. Data from one representative experiment ($n = 6$) with standard errors are shown.

(A)



(B)

1 MTERRVPFSL LRGPSWDPFR DWYPHSR***LFDQAFGLPR***LPE EWSQWLGGSS
51 WPGYVRPLPP AAIESPAVAA PAYSRALSRQ LSSGVSEIRH TADRWR***VSLD***
101 ***VNHFAPDET VK***TKDGVVEI TGKHEERQDE HGYISRCFTR KYTLPPGVDP
151 TQVSSSLSPE GTLTVEAPMP KLATQSNEIT IPVTFESRAQ LGGPEAAKSD
201 ETAAK

(C)

LFDQAFGLPR

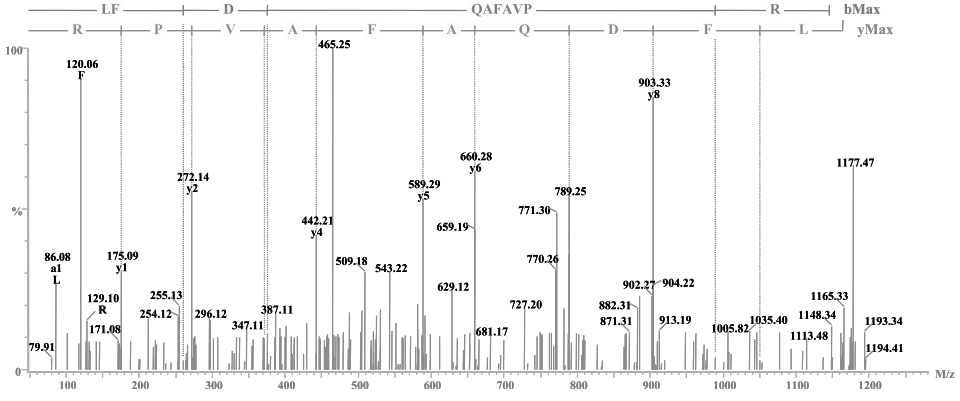


Fig. 4. Identification of a decreased expression of HSP27 via 2-DE followed by MS/MS sequencing. A: Partial 2D images of proteins from cells with and without expanded ataxin-3. The arrows indicate the significantly differential expression of HSP27. B: The peptide identified by tandem mass sequencing is shown underlined within the amino acid sequence of HSP27. C: A MS/MS spectrum of the investigated peptide 582.24 (2+) is shown. The sequence interpretation was performed using y-ions and b-ions. The MS/MS data was matched to HSP27 in the NCBI database using the MASCOT search engine (http://www.matrixscience.com).

antibody against HSP27. A representative Western blot is shown in Fig. 5A. The results demonstrated that the protein levels of HSP27 in the stably transfected cells significantly decreased (Fig. 5A, lane 2) compared with that of the parental cells (Fig. 5A, lane 1). A quantitative assessment of the percentage of protein expression revealed that HSP27 in the stably transfected cells retained only about 20% of that of the parental cells (Fig. 5B). This observation was further confirmed by imunocytochemical staining using mouse monoclonal antibody against HSP-27. As shown in Fig. 6, significant positive staining was observed in the parental cells (Fig. 6A) compared with very weak staining in cells containing expanded ataxin-3 (Fig. 6B). Meanwhile, lymphoblastoid cell lines (LCLs) from two MJD patients and one normal individual were used for comparison. Western blot analysis demonstrated that both normal and mutant ataxin-3 were expressed

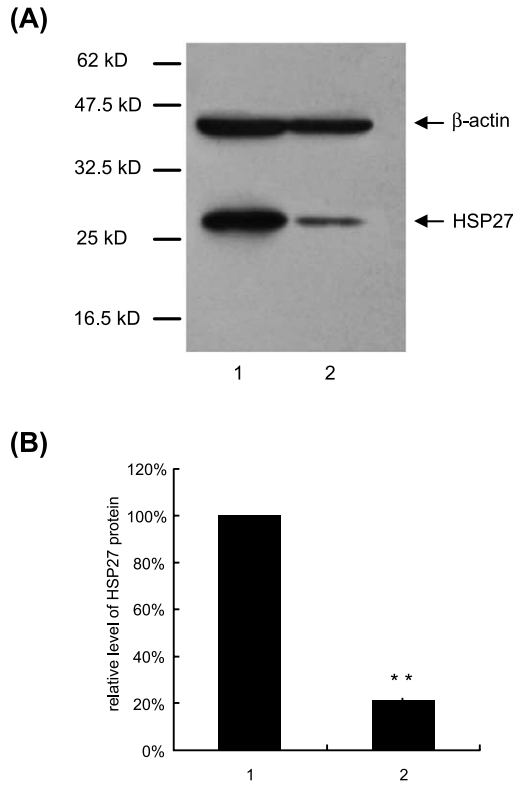


Fig. 5. HSP27 expression in the cells stably transfected with expanded ataxin-3 or the parental cells. A: Western blot analysis of HSP27 proteins. Untransfected SK-N-SH human neuroblastoma cells (lane 1) and the cells stably transfected with expanded ataxin-3 (lane 2) were cultured in complete medium. Cell lysates were electrophoresed, Western blotted and probed with anti-HSP27 antibody and anti-β-actin antibody. B: HSP-27 expression in the stably transfected cells relative to the control. $n=4$; $^{**}P<0.0001$.

in samples from two patients (Fig. 7A, lanes 2 and 3), compared with only one normal ataxin-3 in the control LCL (Fig. 7A, lane 1). We further confirmed a significant reduction of HSP27 in two MJD LCLs (Fig. 7B, lanes 2 and 3), compared with that from a normal individual (Fig. 7B, lane 1). Furthermore, we compared the expression of other HSPs in our neuronal cellular model to understand whether the reduction of HSP27 was due to a common effect on heat shock response. By contrast, other stress associated HSPs, namely HSP60, 70 and 90 examined by Western blot analysis, remained unaffected in SK-N-SH-MJD78 cells (data not shown). These results indicated that the altered protein expression of HSP27 was specific in the cellular model. We next determined whether this reduction in HSP27 was due to transcriptional repression of HSP27 gene. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and microarray analysis indicated that the differences we observed in the protein levels were not due to transcriptional defects (data not shown).

4. Discussion

In the present study, neuronal SK-N-SH-MJD78 cells, stably expressing expanded ataxin-3, were established to analyze the role(s) of mutant ataxin-3 in human neuronal cells. In contrast to transient transfection or inducible polyglutamine expression, cells in the present study did not undergo a rapid form of cell death under basal conditions. The clonal cells expressing expanded full-length ataxin-3 showed less than 1% intranuclear aggregates (Fig. 2C), indicating that the physiological condition of our cellular model was more likely to mimic the early stage of this late-onset disease. Although no increased cell death was observed in the SK-N-SH-MJD78 cells for more than 80 passages, we could not rule out the possibility that cells may have compromised viability. Many mammalian cellular models of poly(Q) disease have shown that overexpression of poly(Q) protein containing a poly(Q) expansion resulted in toxicity/cell death [24,34]. Chemicals and conditions that damage proteins, promote protein misfolding, or inhibit protein processing triggered the onset of protective homeostatic mechanisms resulting in 'stress responses' in mammalian cells. It was reported that rat pheochromocytoma PC12 cells stably expressing polyglutamine expansion were more vulnerable to exogenous stress [34]. In this study, our results demonstrated that cells with expanded ataxin-3 were more susceptible to exogenous oxidative stress than the parental cells, indicating that these cells had weak protection effects upon the extracellular oxidative stress.

In an attempt to determine whether the expression of expanded ataxin-3 altered gene expression that may be important in the neurotoxicity observed in MJD, comparative pro-

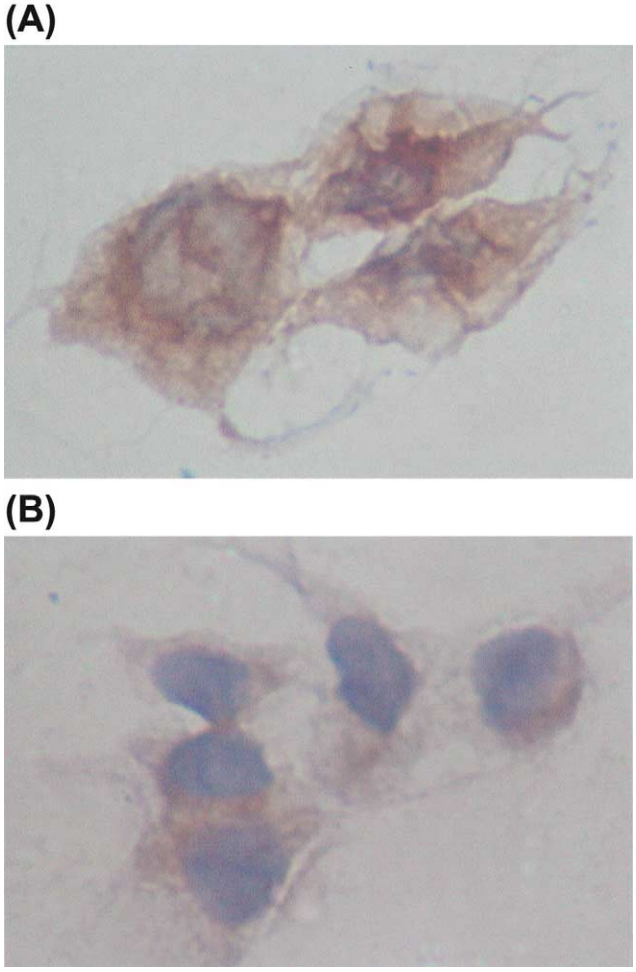


Fig. 6. Immunocytochemical analysis of expressed HSP27. SK-N-SH and (A) SK-N-SH-MJD78 (B) were labeled with monoclonal anti-HSP27 antibodies (magnification: 1000×).

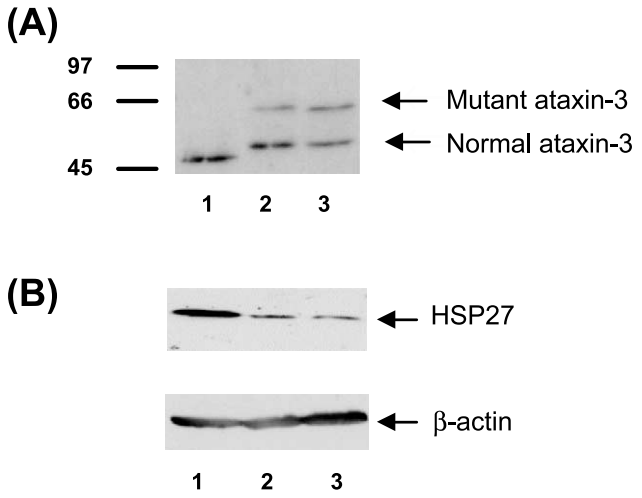


Fig. 7. A: Western blot analysis of ataxin-3 in lymphoblastoid cells from MJD-affected and normal individuals. Normal lymphoblastoid cells (lane 1) and lymphoblastoid cells from two affected individuals (lanes 2 and 3) were cultured in complete medium. Western blot was probed with monoclonal anti-ataxin-3 antibodies. B: Western blot analysis of HSP27 in lymphoblastoid cells from MJD-affected and normal individuals. Normal lymphoblastoid cells (lane 1) and lymphoblastoid cells from two affected individuals (lanes 2 and 3) were cultured in complete medium. Cell lysates were electrophoresed, Western blotted and probed with anti-HSP27 antibody together with anti-β-actin antibody as an internal control.

teome analysis was performed and a dramatic expression difference in HSP27 was identified. Western blots further demonstrated that the significant reduction of HSP27 was observed in both neuronal cells and non-neuronal cells expressing expanded MJD. It ruled out the possibility that the reduction of HSP27 was the result from overexpression of a certain protein in SK-N-SH cells. In addition, it was worthy noting that the reduced HSP27 expression was not due to transcriptional dysregulation, as indicated through semiquantitative RT-PCR and microarray analysis (data not shown). This observation ruled out the possibility that HSP27 gene may be disrupted by the transgene in our stable cell line. It was shown that HSP27 was regulated at different levels along with the expression, including transcriptional, translational and posttranslational [35]. Further studies will be required to address the mechanism underlying the reduced protein level of HSP27 in the disease model.

HSP27 was reported to protect cells against oxidative stress and have anti-apoptotic properties in neuronal survival [16,17,24,36]. The expression of HSP27 led to a decrease in ROS and to an increase in glutathione [36,37]. Recently, it was reported that mutant huntingtin caused increased levels of ROS in neuronal cells and transiently transfected HSP27 suppressed the increased levels of cellular ROS without interfering poly(Q) aggregation [24]. Therefore, it is possible that a reduction of HSP27 in the presence of expanded ataxin-3 resulted in an increase of ROS in the cellular model. However, we cannot rule out the possibility that the reduced level of HSP27 may be involved in other pathway(s) that lead to cell death in the pathogenesis of MJD. It was reported that HSP27 inhibits the mitochondrial death pathway by binding to and inhibiting apoptosome formation [13]. In addition, the phosphorylated dimers of HSP27 were demonstrated to interact with Daxx and prevent the interaction of Daxx with both Ask1 and Fas, therefore blocking Daxx-mediated apoptosis [38]. It is possible that HSP27 has a specific target in a key apoptotic signaling or execution pathway, which was supported earlier by the report that HSP27 can prevent activation of pro-caspase 9 after etoposide treatment and can inhibit apoptosis induced by activation of the Fas receptor [36,39]. Our next works will be addressing which pathway that HSP27 may be involved in the molecular pathogenesis of MJD.

Even though we do not yet understand the mechanism responsible for the significant reduction of HSP27, this observation in the early disease stage suggested that the loss of HSP27 protection activity together along with aging might ultimately lead to cell death. However, we cannot exclude the possibility that other cellular proteins involving signal transduction and/or apoptosis pathways also play important roles in the pathogenesis of MJD. It is important to note that the neuronal cells contained the ability to withstand the existence of expanded ataxin-3 for prolonged periods without apparent adverse effects in our cellular model. It is likely that cells underwent genetic or biochemical changes that allowed them to cope with expanded full-length ataxin-3 in the early stage of the disease. Further analysis of the roles of expanded ataxin-3 will help to better understand its physiological functions and how the poly(Q) expansion in the mutant protein interferes with those and/or other activities.

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