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Identification of DJ-1-Associated Regions on Human Genes from SH-SY5Y Cells using Chromatin Immunoprecipitation Sequence Technique

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Abstract

DJ-1, a cancer- and Parkinson's disease-associated protein, works as a coactivator to various transcription factors. In this study, DNA fragments that bind to DJ-1 complexes were obtained by a chromatin immunoprecipitation sequencing with an anti-human DJ-1 antibody using chromatin from SH-SY5Y cells. We identified 60 different sequences as potential DJ-1 complex-binding sites in genes. Of sequences identified, expression levels of DJ-1-associated site-containing genes for *DNA polymerase N, estrogen receptor α* and *S-adenosylhomocysteine hydrolase like-2* were decreased in DJ-1-knockdown cells and in 6-OHDA-treated cells. These studies suggest that DJ-1 regulates the expression of versatile genes at the transcriptional level and that some of the genes are regulated by DJ-1 in an oxidative status-dependent manner.

Keywords: ChIP sequences; DJ-1; Transcriptional regulation; Oxidative stress; Genome-wide analysis; Cell growth

Abbreviations: ChIP: Chromatin Immunoprecipitation; RT-PCR: Reverse Transcription-PCR; 6-OHDA: 6-Hydroxydopamine

Introduction

DJ-1 was identified by us as a novel oncogene [1] and was later identified as also a causative gene (*park7*) for a familial form of Parkinson's disease [2]. DJ-1 has multiple functions, including transcriptional regulation, anti-oxidative stress function, functions as a chaperone and protease and mitochondrial regulation [3-5]. For transcriptional regulation, DJ-1 acts as a coactivator that binds to various transcription factors, including inhibitors of the androgen receptor [6-8], p53 [9-11], polypyrimidine tract-binding Protein-associated Splicing Factor (PSF) [12], Keap1, an inhibitor of nuclear factor erythroid-2 related factor 2 [13], sterol regulatory element binding protein (SREBP) [14] and RREB1 [15], and regulates their transcriptional activity, resulting in various effects on signaling pathways, cell cycle movement, oxidative stress reaction and dopamine synthesis. It is therefore thought that loss of and excess activation of DJ-1 lead to the onset of neurodegenerative diseases such as Parkinson's disease and cancer [16-21], respectively. Only a few genes regulated by DJ-1, however, have been identified.

Chromatin immunoprecipitation (ChIP) assays are used to identify a transcription factor that binds to specific regions in genes of interest. For genome-wide screening of transcription factors and for identification of their recognition sequences on genomes, the ChIP technique has been applied to next-generation DNA sequencers and this technique is named ChIP sequencing [22-24].

In this study, we screened DJ-1 complex-binding sites in the genome of human SH-SY5Y cells by the ChIP sequencing and obtained 60 different sequences, including sequences upstream of the *POLN* gene and in introns of *ESR1* and *AHCYL2* genes. We also found that the expression levels of *POLN, ESR1* and *AHCYL2* genes were decreased in DJ-1-knockdown cells and that the expression levels and a number of DJ-1-associated sites were decreased in cells under oxidative conditions. These results suggest that DJ-1 regulates expression of versatile genes at the transcriptional level and that some of the genes are regulated by DJ-1 in a DJ-1 oxidative status -dependent manner.

Materials and Methods

Cell culture

Human SH-SY5Y and mouse NIH3T3 cells were purchased from American Type Culture Collection. DJ-1-knockdown SH-SY5Y cells (about 50% knockdown of DJ-1 expression) [25] and DJ-1-knockdown NIH3T3 cells (D2 cells) (about 40% knockdown of DJ-1 expression) [26] were established previously. DJ-1-knockdown NIH3T3 cells (D2 cells) were well-characterized and used in transcriptional regulation and gene expression studies of DJ-1 [14,15,26-28]. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2.

Chromatin immunoprecipitation (ChIP) and sequence analysis

5×10^7 SH-SY5Y cells were treated with 50 μM 6-OHDA for 48 hrs and cross-linked with formaldehyde. DNA-protein complexes were then prepared from SH-SY5Y cells and from 6-OHDA-treated SH-SY5Y cells as described previously [6]. ChIP assays were carried out with a rabbit anti-human DJ-1 polyclonal antibody or with non-specific IgG using a ChIP assay kit (Upstate) according to the manufacturer's protocol. The rabbit anti-human DJ-1 polyclonal antibody described previously [1] was affinity-purified using a DJ-1-coupled sepharose resin. For ChIP sequences, adaptors (Illumina) were ligated to immunoprecipitated DNAs and their sequences were determined using Genome Analyzer II (GAII, Illumina). Of total 7,702,242 and 5,814,987

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short reads that had been ChIP-sequenced in samples from SH-SH5Y cells and 6-OHDA-treated SH-SY5Y cells, 1,849,642 and 2,227,434 reads, respectively, were mapped to the human genome (UCSC hg 18, excluding haplotype sequences) by using ELAND from Illumina data analysis software, which maps sequences within 2 mismatches.

ChIP assays using cultured SH-SY5Y cells were performed according to the protocol of the ChIP Assay Kit (Millipore, Billerica, MA, USA). Briefly, after proteins had been cross-linked with DNA, cell pellets were resuspended in an SDS-lysis buffer and sonicated on ice using a sonicator (UR-20P, TOMY, Tokyo, Japan) 3 times for 20 sec each time. Genomic DNA was then sheared to 300 to 1200 base pairs of length. Chromatin solution from 1×10^6 cells/dish was preincubated with salmon sperm DNA and Protein A-agarose and incubated with species-matched IgG or with specific antibodies overnight at 4°C. DNA fragments immunoprecipitated were then used as templates for PCR with Ex taq (TaKaRa Bio, Kyoto, Japan) and reacted for 1 min at 94°C, 34-37 cycles of 0.5 min at 94°C and 0.5 min at 72°C. Nucleotide sequences of oligonucleotide used for ChIP primers were as follows: ESCO1ChIP-F: 5'-GCTAAGATGACACCGCAACA-3', ESCO1ChIP-R:5'-GTCAGGCTGGTCTCGAACTC-3', POLNChIP-F:5'-AAAGACTGGGTGGGAGGAGT-3', POLNChIP-R:5'-CCCCTCAGCTTGTGTTGTTTT-3', ESR1ChIP-F: 5'-TGGGCCCTTAATCTAATGTGA-3', ESR1ChIP-R: 5'-TTCCTAGGCACCAGCAATCT-3', AHCYL2ChIP-F: 5'-GTCCAGAGGATTGCTTGAGG-3' and AHCYL2ChIP-R: 5'-GCCTCAGCTGTCATGTCCTT-3'. PCR products were separated on 2% agarose gels and stained with ethidium bromide. Reverse images of black and white staining in semi-quantitative RT-PCR are shown.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR

Total RNAs were prepared from cells using an RNeasy mini kit (Qiagen) and their quality was examined using Bioanalyzer (Agillent). Five hundred ng of total RNAs was used for reverse transcription using Superscript III (Invitrogen). Nucleotide sequences of forward and reverse primers in RT-PCR and real-time PCR are shown in Table 1. PCR was carried out with HS taq polymerase (Hokkaido System Science Co. Ltd.) and PCR conditions were as follows: 15 min at 96°C, 32-40 cycles of 30 sec at 96°C, 30 sec at 60°C and 30 sec at 72°C, and 5 min at 72°C. After reactions, PCR products were extracted, separated on 2% agarose gels, and stained with ethidium bromide. Intensities of bands were quantified using ImageJ software. β-actin mRNA was also amplified as a control. Real-time PCR was carried our as described previously [25]. Real-time PCR conditions were as follows: 3 min at 94°C, 39 cycles of 30 sec at 94°C and 30 sec at 60°C.

Human		Mouse	
Primer name	Sequence (5'---3')	Primer name	Sequence (5'---3')
ESCO1 3198F	cctggtgctgctcaacatta	ESCO1 2109F	tgcgctctaatcggttcttt
ESCO1 3396R	tgtgttgcaaacagctttcc	ESCO1 2332R	ggacactggatgaggcattt
GPHN2944F	ccatgggggaaaaggactat	GPHN1955F	ccatgggggaaaaggactat
GPHN3147R	gtgcaggcacaacaaagaga	GPHN2114R	ggattccctggtagtgcaaa
POLN 1559F	atgctctgcgagaccttcat	POLN 115F	taccctctctgctgttgct
POLN 1767R	aatctgaattgggtgcttgg	POLN 294R	actcggggttctttgttcct
ESR1 1624F	agcaccctgaagtctctgga	ESR1 2891F	aagggcagtcacaatgaacc
ESR1 1776R	gatgtgggagaggatgagga	ESR1 3045R	gccaggtcattctccacatt
AHCYL2 476F	gtcgctctttgtctcgttcc	AHCYL2 3518F	gtgccctggaagctagtctg
AHCYL2 684R	tgcaggcatttcttgttcag	AHCYL2 3714R	acggcctatctcggtaaggt
RELB 1924F	tcccaaccaggatgtctagc	RELB 1405F	tgatccacatggaatcgaga
RELB 2083R	agccatgtccctttcctct	RELB 1556R	caggaagggatatggaagca
DJ-1 299F	tgtagccgtgatgtggtcat	DJ-1 649F	gcaccgcttgttctcaaag
DJ-1 511R	ttcatgagccaacagagcag	DJ-1 899R	tggcaggagcttggtaaact
ACTB 875F	cttcctgggcatggagtc	ACTB 412F	ccctaaggccaaccgtgaaa
ACTB 952R	ggatgtccacgtcacacttc	ACTB 520R	acgaccagaggcatacaggga

Table 1: Nucleotide sequences of primers used for RT-PCR and real-time PCR.

Statistical analyses

Statistical analyses were carried out using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test, and data are expressed as means ± S.D.

Results and Discussion

Identification of DJ-1-targeting genes in SH-SY5Y cells

ChIP-sequencing was then carried out to identify potential DJ-1 binding/recognition sites in cells using GAII, and mapping of DJ-1 associated/recognition sites in genes was carried out using UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg18) Assembly. Mapping peaks on human genome were detected using Illumina Genome studio ChIP sequence module ver.1.0, and DNA sequences that had been immunoprecipitated with the anti-DJ-1 antibody but not with IgG were mapped. Since it is not clear whether DJ-1 directly binds to DNA and since it has been reported that DJ-1 acts as a coactivator by binding to various transcription factors that possess DNA-binding activity, it is thought that DJ-1 or DJ-1 complex recognizes specific sequences in respective genes. In this study, we tentatively call these sites "DJ-1-associated sites" for convenience.

We found 60 potential DJ-1-binding sites with different sequences in human genome and that their mapping numbers on chromosomes 18, 19, 7 and 4 were 3024, 80, 73 and 71, respectively .For instance, two peaks corresponding to DJ-1-associated sites, peaks a and b that are located in regions 16,767,578-16,767,618 and 17,387,379-17,387,415 on chromosome 18, were detected, and their mapping number was 62 and 3024, respectively (Table S1). Regions of peaks a and b were then found to be located downstream and in intron of genes encoding Rho-associated Coiled-coil Containing protein Kinase 1 (ROCK1) and Establishment of Cohesion 1 (ESCO1), respectively. CLUSTAL W (1.83) Multiple Sequence Alignments were then used to alien sequences obtained. Aliened sequences were, however, poly A stretch and AG repeat but not specific sequences. Since DJ-1 binds to DNA via other DNA-binding transcription factors, it is reasonable to have identified variety of different sequences as DJ-1-binding sequences. Nucleotide sequences identified in this study have been deposited to the NCBI database, and its accession number is DRA000365.

Reduced expression of the establishment of cohesion 1 (ESCO1) gene in DJ-1 knockdown cells

Of the genes identified, the highest hit of DJ-1-binding sites in the ChIP sequence was placed in intron of the Establishment of Cohesion 1 (ESCO1) gene on chromosome 18. To confirm the binding activity of DJ-1 to the *ESCO1* gene, ChIP assays were carried out using chromatin from SH-SY5Y cells and an anti-DJ-1 antibody or non-specific IgG. As shown in Figure 1, the anti-DJ-1 antibody but not IgG precipitated the *ESCO1* gene spanning +27109 to +27367. To examine the relationship between the *ESCO1* gene and DJ-1, total RNA was extracted from parental and knockdown cells of human SH-SY5Y and mouse NIH3T3 cells, and the expression levels of *ESCO1*, DJ-1 and β-actin (ACTB) mRNA were examined by semi-quantitative RT-PCR. ACTB mRNA was used as a loading control. As shown in (Figures 2A and C), expression levels of ESCO1 mRNA in DJ-1- knockdown cells of NIH3T3 and in SH-SY5Y cells were reduced to about 80% and 40%, respectively, of those in parental NIH3T3 cells and in SH-SY5Y cells.

Expression levels of the *ESCO1* gene in parental and its knockdown human and mouse cells were also examined by quantitative real-time PCR. Results again showed reduced expression of the ESCO1 gene in DJ-1-knockdown cells (Figures 2B and 2D).

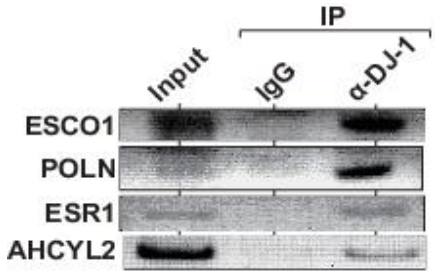

Figure 1: Confirmation of DJ-1-associated to genes that had been identified by ChIP sequences.
Chromatin immunoprecipitation assays were carried out using chromatin prepared from SH-SY5Y cells. Chromatin was immunoprecipitated with anti-DJ-1 or non-specific IgG. After extraction of DNA from precipitated chromatin, regions spanning +27109 to +27367, -84923 to -84642, +42384 to +42619 and +108161 to +108379 in ESCO1, POLN, ESR1 and AHCYL2 genes, respectively, were amplified by PCR with specific primers as described in Materials and methods. Reverse images of black and white staining in PCR are shown

Frequency of DJ-1-associated sites and expression levels of genes under an oxidative stress condition

The frequency of potential DJ-1-associated sites mapped was changed after SH-SY5Y cells had been treated with 50 μM 6-OHDA for 48 hrs. As shown in Table 2, five fragments were decreased by more than 7 fold compared to those in SH-SY5Y cells without 6-OHDA treatment. To first examine whether the expression of these genes is regulated by DJ-1 under normal conditions, total RNAs were extracted from NIH3T3 and D2 cells and the expression levels of GPHN, POLN, ESR1, AHCYL2, RELB, DJ-1 and ACTB mRNA were examined by RT-PCR. ACTB mRNA was used as a loading control. As shown in Figure 3A, expression levels of *GPHN, POLN, ESR1* and *AHCYL2* genes were significantly decreased, while expression level of the *RELB* gene was not changed in D2 cells. Expression levels of *GPHN, POLN, ESR1* and *AHCYL2* genes were further examined using DJ-1-knokcdown SH-SY5Y cells. As shown in Figures 3B, expression levels of *POLN, ESR1* and *AHCYL2* genes were significantly decreased and expression level of the *GPHN* gene was not changed. Since expression levels of *POLN, ESR1* and *AHCYL2* genes were significantly reduced in DJ-1-knockdown cells of both NIH3T3 and SH-SY5Y cells, these genes were further examined by real-time PCR, and significant reduction of their expression levels in DJ-1-knokcdown SH-SY5Y cells was again observed (Figures 3C).

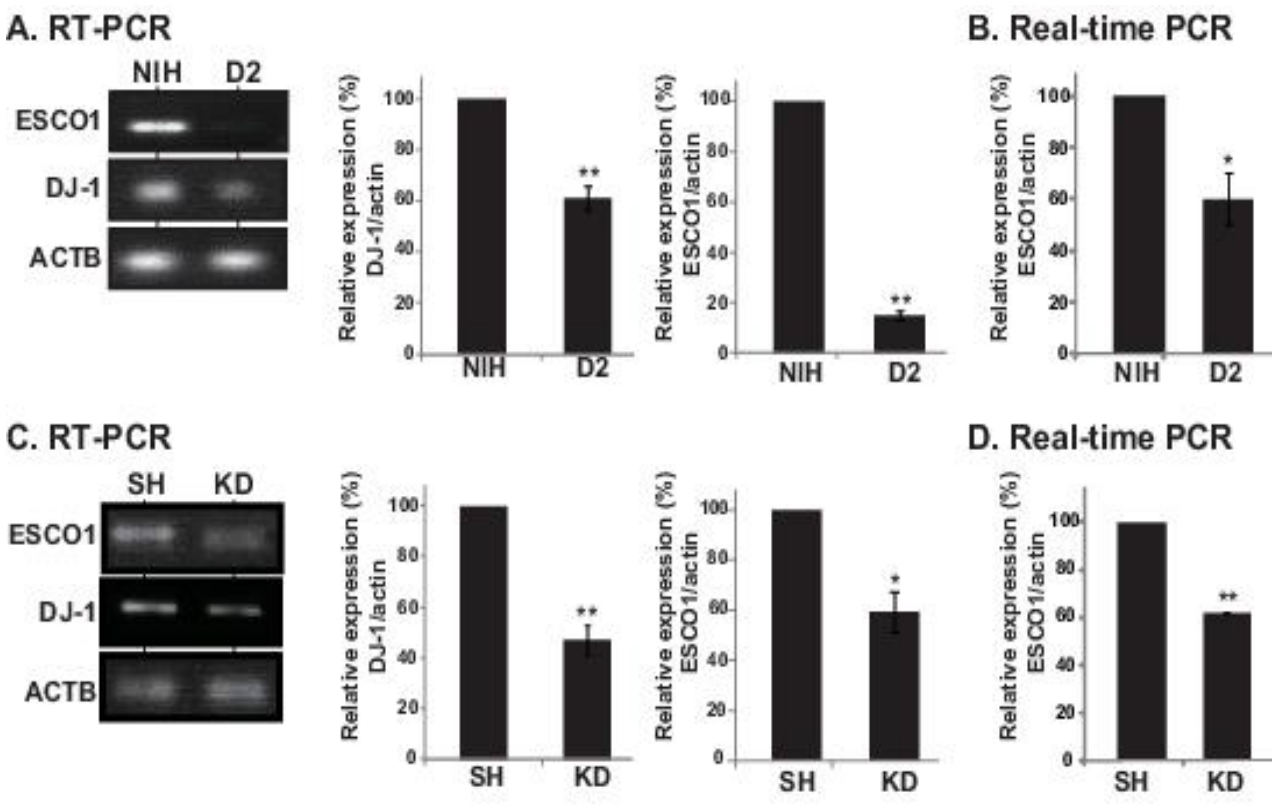

Figure 2: Expression of the ESCO1 gene in presence and absence of DJ-1.
A. Expression levels of ESCO1, DJ-1 and β-actin (ACTB) mRNA in mouse NIH3T3 and its DJ-1-knockdown D2 cells were examined by RT-PCR and quantified (left and right panels, respectively). Relative expression levels of ESCO1 and DJ-1 to the level of ACTB are shown. Reverse images of black and white staining in RT-PCR are shown.
B. Expression levels of ESCO1, DJ-1 and β-actin (ACTB) mRNA in mouse NIH3T3 and its DJ-1-knockdown D2 cells were examined by real-time PCR. ***p*<0.01: D2 cells vs. NIH3T3 cells.
C. Expression levels of ESCO1, DJ-1 and ACTB mRNA in human SH-SY5Y and its DJ-1-knockdown cells were examined by RT-PCR and quantified (left and right panels, respectively). Reverse images of black and white staining in RT-PCR are shown.
D. Expression levels of ESCO1, DJ-1 and ACTB mRNA in human SH-SY5Y and its DJ-1-knockdown cells were examined by real-time PCR.
***p*<0.01: knockdown cell vs. SH-SY5Y cells.

	Peak	Control/6-OHDA (-fold)	Gene Name	Position
Chromosome 4	a	11.6	*POLN*	upstream
Chromosome 6	g	7.4	*ESR1*	on intron
Chromosome 7	c	8.3	*AHCYL2*	on intron
Chromosome 14	a	16.4	*GPHN*	on intron
Chromosome 19	b	8.2	*RELB*	downstream

More than 7 fold changes in DJ-1-binding sites in SH-SY5Y cells treated with 6-OHDA compared to those in untreated SH-SY5Y cells are shown

Table 2: Mapping number of DJ-1-binding sites after SH-SY5Y cells has been treated with 6-OHDA.

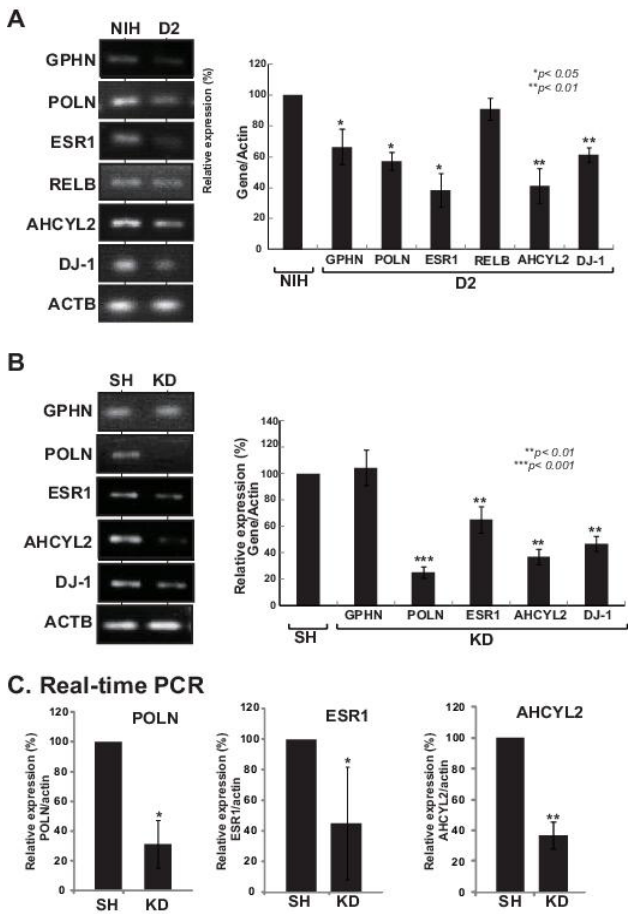

Figure 3: Expression of genes containing DJ-1-associated sites in DJ-1-knockdown cells.

A. Expression levels of ESCO1, GPHN, POLN, ESR1, RELB, AHCYL2, DJ-1 and ACTB mRNA in mouse NIH3T3 and its DJ-1-knockdown D2 cells were examined by RT-PCR and quantified (left and right panels, respectively). Relative expression levels of genes to the level of ACTB are shown. *p<0.05 and **p<0.01: D2 cells vs. NIH3T3 cells. Reverse images of black and white staining in RT-PCR are shown.

B. Expression levels of ESCO1, GPHN, POLN, ESR1, AHCYL2, DJ-1 and ACTB mRNA in human SH-SY5Y cells and its DJ-1-knockdown cells were examined by RT-PCR and quantified (left and right panels, respectively). Reverse images of black and white staining in RT-PCR are shown.

C. Expression levels of ESCO1, POLN, ESR1, AHCYL2 and ACTB mRNA in human SH-SY5Y cells and its DJ-1-knockdown cells were examined by real-time PCR. Relative expression levels of genes to the level of ACTB are shown. **p<0.01 and ***p<0.001: knockdown cells vs. SH-SY5Y cells.

Furthermore, binding activity of DJ-1 to *POLN, ESR1* and *AHCYL2* genes were confirmed by ChIP assays using chromatin from SH-SY5Y cells and an anti-DJ-1 antibody (Figure 1).

To examine the effect of oxidative stress and DJ-1 on expression of *POLN, ESR1* and *AHCYL2* genes, total RNAs were extracted from SH-SY5Y cells treated with or not treated with 6-OHDA, and expression levels of these mRNAs were examined by semi-quantitative RT-PCR and by quantitative real-time PCR. It was first confirmed that expression levels of *POLN, ESR1* and *AHCYL2* genes were reduced in DJ-1-knockdown SH-SY5Y cells that had been treated with 6-OHDA compared to those in non-treated DJ-1-knockdown SH-SY5Y cells (Figure 4C), indicating that treatment of 6-OHDA did not affect the positive effect of DJ-1 on the expression of these genes. As shown in Figures 4A and 4B, the expression levels of POLN and AHCYL2 mRNA in 6-OHDA-treated SH-SY5Y cells were reduced to about 40-50% and 78-60%, respectively, of that in untreated SH-SY5Y cells by analysis of RT-PCR and real-time PCR. The expression level of ESR1 mRNA, on the other hand, was not changed, rather increased, after cells had been treated with 6-OHDA. Since the expression levels of these genes were reduced in DJ-1-knockdown cells and since the expression levels of *POLN* and *AHCYL2* genes but not that of the *ESR1* genes were reduced in SH-SY5Y cells that had been treated with 6-OHDA, these results suggest that DJ-1 regulates gene expression in an oxidative stress-dependent or independent manner.

In this study, we newly found 60 potential DJ-1-associated/recognizing sites in human genes by ChIP sequencing using a next-generation DNA sequencer. DJ-1-associated sites were found to be located upstream, in introns and downstream of coding regions of genes that cover many genes possessing versatile functions. Of the DJ-1-associated sites identified, the highest mapping score was obtained in the intron of the establishment of cohesion 1 (*ESCO1*) gene, and the expression level of *ESCO1* mRNA was decreased in DJ-1-knockdown cells of human SH-SY5Y and mouse NIH3T3 cells, suggesting that the ESCO1 gene is regulated by DJ-1 at the transcriptional level under a non-stressed condition. ESCO1 is required for proper sister chromatid cohesion. Although there is no evidence at present, DJ-1 might control the segregation of sister chromatids.

Furthermore, we found that the number of potential DJ-1-associated sites in human genome was changed after cells had been treated with 6-OHDA. DJ-1-associated sites identified are regions upstream of the DNA polymerase N (*POLN*) gene, downstream of the Estrogen Receptor α (*ESR1*) gene and in the intron of the Adenosylhomocysteine Hydrolase-like 2 (*AHCYL2*) gene, and expression levels of these genes were significantly decreased in DJ-1-knockdowned SH-SY5Y cells before and after treatment of the cells with 6-OHDA, indicating that DJ-1 positively regulates the expression of these genes regardless of oxidative stress. While expression levels of *POLN* and *AHCYL2* genes were also decreased in SH-SY5Y cells treated with 6-OHDA compared to those in cells without 6-OHDA treatment, expression of the *ESR1* gene was not changed after oxidative stress. Cysteine residues, especially cysteine at amino acid number 106 (C106), of DJ-1 are oxidized in cells treated with 6-OHDA and the oxidative status of C106 regulates DJ-1's activity [29-31]. Since both the frequency of DJ-1-binding sites detected by a ChIP sequencing and the expression levels of *POLN* and *AHCYL2* genes were decreased in SH-SY5Y cells that had been treated with 6-OHDA, it is thought that reduced or weakly oxidized DJ-1 binds to the DJ-1-recognition sites in *POLN* and *AHCYL2* genes but that highly oxidized DJ-1 does not, resulting in reduction of their gene expression in 6-OHDA-treated SH-SY5Y cells. Expression level of the *ESR1* gene, on the other hand, was reduced in DJ-1-knockdown cells but not in cells treated with 6-OHDA, suggesting that DJ-1 positively regulates the ESR1 gene under a non-oxidative stress condition.

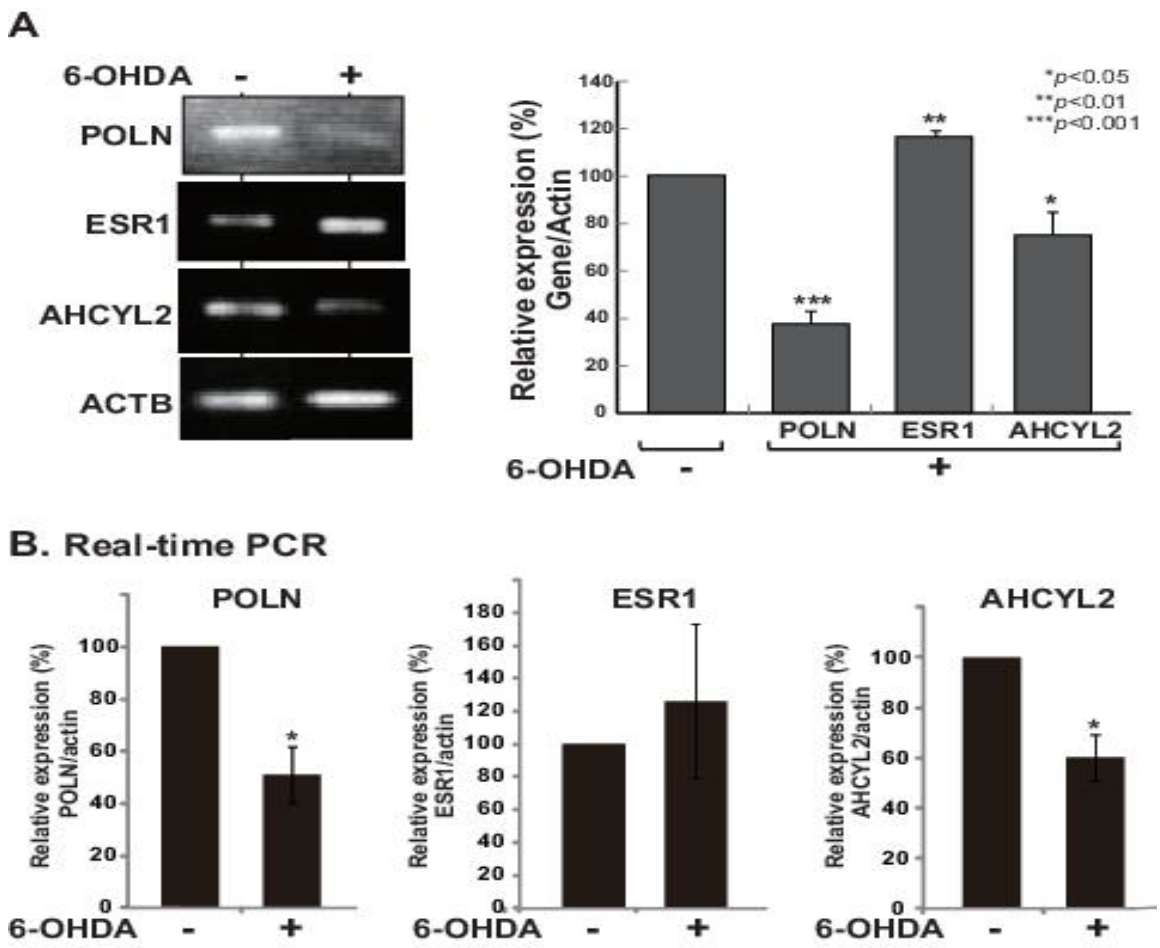

Figure 4: Expression of genes containing DJ-1-associated sites in cells treated with 6-OHDA.
Expression levels of POLN, ESR1, AHCYL2, DJ-1 and ACTB mRNA in human SH-SY5Y and its DJ-1-knockdown cells that had been treated with 6-OHDA were examined by RT-PCR and quantified (A, left and right panels, respectively), and by real-time PCR (B). Expression levels of POLN, ESR1, AHCYL2, DJ-1 and ACTB mRNA in DJ-1-knockdown SH-SY5Y cells that had been treated with 6-OHDA were examined by RT-PCR and quantified (C). Relative expression levels of genes to the level of ACTB are shown. *p<0.05, **p<0.01 and ***p<0.001: knockdown cells vs. SH-SY5Y cells.

Conclusions

In conclusion, expressions of *ESCO1, POLN, ESR1* and *AHCYL2* genes are regulated by DJ-1 to protect cells against oxidative stress-induced onset of diseases such as Parkinson's disease. These findings revealed new target genes regulated by DJ-1. It would be interesting to further analyze the effects of DJ-1 on segregation of sister chromatids, DNA replication through the ESCO1, ROS-generated translesion synthesis through POLN and 17beta-estradiol-exerting protective action against ischemic injury through ESR1, and metabolism of homocysteine through AHCYL2.

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