

Table 2. List of genes having the same tendency of expression level in most twin pairs.

Up-regulated in co-twins with	Gene	Shared by twin pair ID
Higher IQ scores	<i>GTSF1</i>	1,2,7,11,14,15,16
	<i>AK3L1</i>	1,3,9,11,17
	<i>PRKCH</i>	2,9,11,14,17
	<i>CDRT1</i>	2,9,10,11,14
	<i>LRIG3</i>	1,2,9,15
	<i>VSIG6</i>	2,10,11,13
	<i>SNORA20</i>	3,6,10,12
	<i>UCHL1</i>	8,9,11,15
Lower IQ scores	<i>CD96</i>	1,2,4,9,14
	<i>CXCL10</i>	1,6,9,11
	<i>CDC42BPA</i>	1,2,6,9
	<i>CXCR4</i>	1,2,6,14
	<i>EPS8</i>	2,9,10,16
	<i>FAM169A</i>	3,9,11,15

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Promoter 1.0 Array contains 4.6 million probes). As a result, we were able to detect more candidate loci from the practically congruent MZ twin samples, only at the expense of precision rate. The technical limitations of MethylMiner collection might also contribute to false positives. After bisulfite sequencing and qRT-PCR validation, we identified one candidate gene, *ARHGAP18*, which encodes one of the Rho GTPase-activating proteins (GAPs) that modulate cell proliferation, migration, intercellular adhesion, cytokinesis, proliferation differentiation, and apoptosis [25]. Mutations in a handful of Rho-linked genes were documented to

be associated with X-linked mental retardation [8], by which the importance of GAP activity in normal neuronal functions was proposed [26]. Notwithstanding the identification of *ARHGAP18* in a genome-wide association study for schizophrenia [27], it had not been previously connected to cognitive abilities until the present study.

We were not able to identify a single gene that displayed significantly different expression level between the group of twins with higher IQ scores and their co-twins. From the list of candidate genes generated by direct pair-wise comparison, *UCHL1* is a brain-specific de-ubiquitinating enzyme. While the substrates are still unknown, loss of its enzyme activity has been reported in neurological diseases such as Alzheimer's disease and Parkinson's disease [28]. In a different approach, *RFK*, *RPL12*, and *RMRP* showed borderline significance. *RFK* encodes riboflavin kinase, an essential enzyme to form flavin mononucleotide, is important in a wide range of biological metabolisms [29]. *RPL12* encodes a ribosomal protein of the 60S subunit, while *RMRP* encodes the RNA component of mitochondrial RNA processing endoribonuclease. Although none of these 3 genes had ever been connected with cognitive functions, it remains possible that their biophysical characteristics might become more pronounced in cells having as high a metabolic rate as neurons.

In the gene set based approach, GSEA of between-group and between co-twin comparisons revealed several mitochondrial ribosomal protein-coding genes. Mitochondria, which are responsible for most of the energy requirement for cellular metabolism, have their own translation system for the 13 proteins essential for oxidative phosphorylation in mammals. All 78 human mitochondrial ribosome proteins are translation products of nuclear genes, of which some were identified as candidate genes for several congenital diseases [30]. No exclusive conclusion about the connection of mitochondrial ribosomal function and cognitive ability could be drawn before being further validated. Nevertheless, we hypothesized that, for the highly differentiated and high energy-demanding central nervous system, essential proteins for

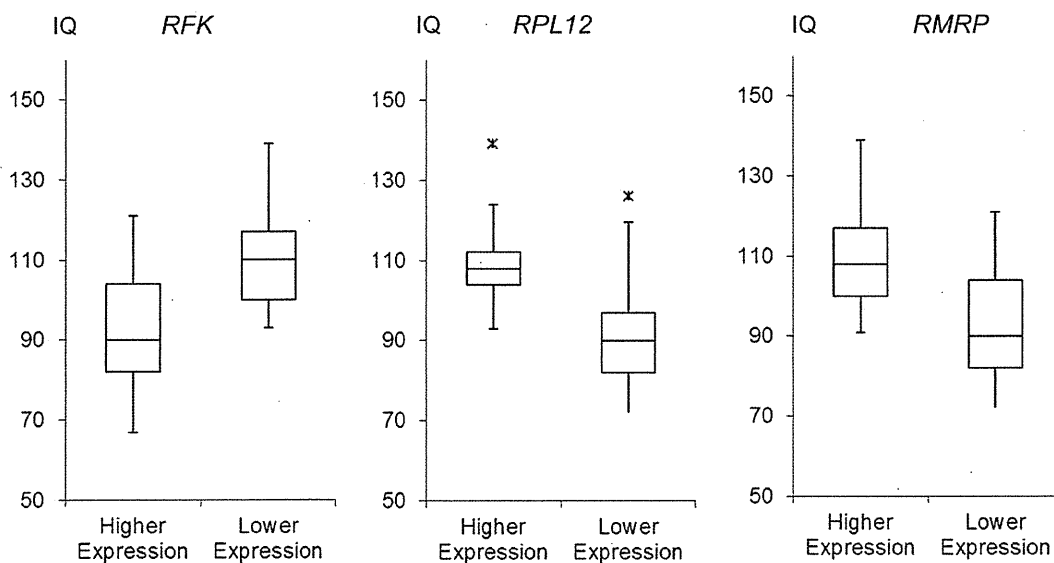


Figure 2. Three genes manifesting borderline significance by grouping samples according to individual gene expression level. IQ scores in subjects were grouped according to the relatively higher (Higher Expression) or lower (Lower Expression) expression levels within each twin pair. Three genes, *RFK*, *RPL12*, and *RMRP*, manifested the smallest p yet failed to meet the cutoff value of $p = 10^{-6}$. Data are presented as box plots (minimum, 25% quartile, median, 75% quartile, maximum). The red asterisks indicate maximum outliers. doi:10.1371/journal.pone.0047081.g002

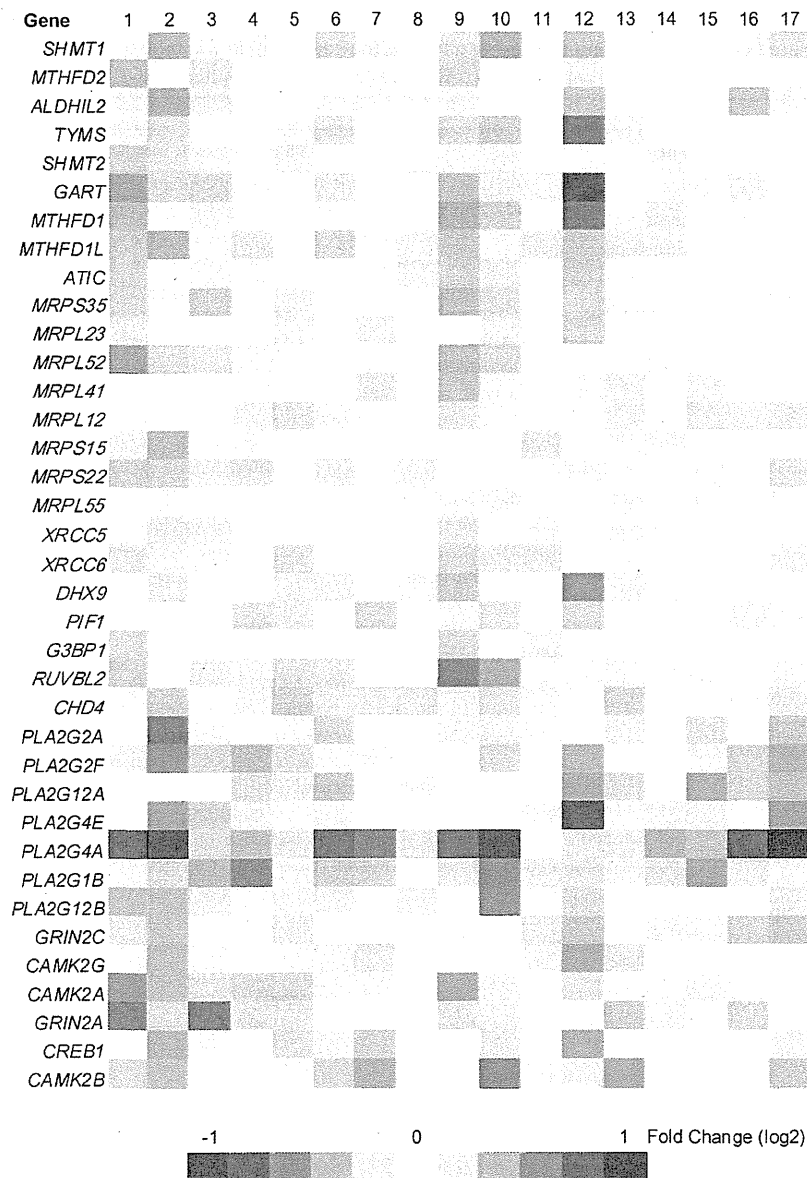


Figure 3. Heat map of the 37 genes with core enrichment from up-regulated genes sets. GSEA analysis was carried out to identify if any pre-defined gene set showing different expression levels between the group of higher IQ twins and the group of their lower IQ co-twins. Gene set databases BioCarta, KEGG, Reactome, and Gene Ontology were applied separative. Gene sets meeting the cutoff FDR q -value of 0.25 were subjected to leading edge analysis to determine the genes with core enrichment. The result was a list of 37 genes and we generated a heat map accordingly. Red and blue cells signify genes that were either up- or down-regulated, respectively, after the expression levels of the twins with higher IQ scores compared to their co-twins. The scale represents fold changes in log₂ values, according to the color map at the bottom of the figure. The general tendency of higher expression levels in twins with higher IQ scores from the gene *SHMT1* to *CHD4*, and their lower expression levels from the gene *PLA2G2A* to *CAMK2B*, was visualized.
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mitoribosome function might play a role in the maintenance of neuronal biological processes.

Apart from mitochondrial ribosomal protein-related gene sets, “ATP-dependent DNA Helicase Activity” from the GO database was also found. DNA helicases are molecular motor proteins that use nucleoside 5'-triphosphate hydrolysis as a source of energy to open energetically stable duplex DNA into single strands. As such, they are essential in almost all aspects of cellular DNA machinery including DNA replication, repair, recombination, and transcrip-

tion [31]. Of which, *XRCC5* and *XRCC6* encode the two subunits of the Ku protein, which plays an important role in the repair of double-stranded DNA breaks and telomere protection [32]. In neurodegenerative diseases, such as Alzheimer's disease, where cellular damage due to oxidative stress is proposed to contribute to pathophysiology, reduced Ku protein expression and its DNA binding activity have been thought to be involved [33]. Of the remaining five helicases denoted, *G3BP1* was demonstrated to play an essential part in proper embryonic growth and neonatal

Table 3. Summary of the most commonly shared gene sets showing the same tendency (up-regulated in the higher IQ twin or in the lower IQ twin) according to the pair-wise GSEA test.

Up-regulated in	Gene sets database	Gene set	Shared by twin pair ID	
Higher IQ twins	BioCarta	MCM pathway	1,9,12,17	
		KEGG	Proteasome	1,3,9,10,17
			Oxidative phosphorylation	1,9,10,12,16
			Ribosome	1,6,9,10,12,16
			DNA replication	1,3,8,9,10,12,17
			Valine leucine and isoleucine degradation	1,3,10,12,17
			Mismatch repair	1,3,9,12,17
			Peroxisome	1,9,10,12,17
		Reactome	RNA polymerase I promoter opening	1,3,9,11,12,13,16,17
			Electrotransport chain	1,3,9,10,12,13,16,17
			Packaging telomere ends	1,3,9,11,12,16,17
		Gene Ontology	Telomere maintenance	1,3,9,11,12,16,17
			Structural constituent of ribosome	9,10,12,17
			Microbody	1,9,12,17
			Peroxisome	1,9,12,17
			S-phase of mitotic cell cycle	1,8,15,17
		Small conjugating protein specific protease activity	1,9,15,17	
Lower IQ twins	BioCarta	n/a	n/a	
	KEGG	Neuroactive ligand receptor interaction	1,3,16,17	
		Taste transduction	1,3,9,13,17	
	Reactome	Cell-cell adhesion systems	1,3,9,17	
	Gene Ontology	Anion transport	1,5,16,17	
		Cation channel activity	1,9,16,17	
		Cell-cell signaling	1,9,16,17	
		Collagen	1,9,16,17	
		Extracellular matrix part	1,9,16,17	
		Extracellular region part	1,9,16,17	
		G-protein coupled receptor protein signaling pathway	1,9,16,17	
		Gated channel activity	1,9,16,17	
		Intercellular junction	6,9,16,17	
		Metal ion transmembrane transporter activity	1,9,16,17	
		Second messenger mediated signal	1,9,16,17	

n/a indicates no gene set was shared by at least 4 twin pairs.
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survival [34]. Although the direct associations between these seven genes and cognitive abilities have not been depicted, mutations in a list of DNA repair-related genes have already been reported to cause mental retardation [8]. As such, one hypothesis we proposed here is that the up-regulated expression of these helicases might provide better protection from oxidative damages and, thus, improve neuronal function and survival, which could bring forth higher levels of intelligence (or in other words, less compromised) as a phenotype.

On the other hand, the pair-wise GSEA, along with leading edge analysis, identified *CHRNA2* which encodes the $\alpha 2$ subunit of nicotinic acetylcholine receptors (nAChRs). Initially related to nicotine dependence, the role of nAChRs in cognitive performance has gained attention because nicotine is considered a powerful enhancer of cognitive capabilities [35] via the interaction of nicotine and nAChRs [36].

Additionally, two potassium voltage-gated channel-coding genes, *KCNE2* and *KCNQ3*, were identified. Voltage-gated ion channels possess diverse functions, include regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, and smooth muscle contraction. By assembling with *KCNQ2* or *KCNQ5*, *KCNQ3* forms the M channel, a slow activating and deactivating potassium channel that plays a critical role in the regulation of neuronal excitability [37]. In addition to being identified as one cause for a dominantly inherited form of human generalized epilepsy, called benign familial neonatal convulsions, the electrogenic characteristics of *KCNQ/M* channels have importance in controlling intrinsic firing patterns of principal hippocampal neurons, thus, further modulating hippocampal learning and memory [38]. Of note, rats treated with Linopirdine, an M channel-specific inhibitor,

Table 4. Up-regulated genes with core enrichment shared by co-twins of multiple pairs.

Up-regulated in	Gene Sets database	Gene	Shared by twin pair ID		
Higher IQ twins	BioCarta	<i>CASP6</i>	1,10,12,17		
		<i>CCNE1</i>	1,9,12,17		
		<i>IGF1</i>	1,2,7,17		
		<i>IL1a</i>	2,7,8,15		
		<i>LTA</i>	2,8,15,17		
		<i>C7</i>	2,3,8,15		
		<i>IL6</i>	2,3,7,15		
		<i>STAT4</i>	3,8,10,17		
		KEGG	<i>MOM6</i>	1,3,8,9,12,17	
			<i>RPA2</i>	1,3,8,9,12,17	
			<i>POLA2</i>	1,3,8,9,12	
			<i>PRIM2</i>	1,3,8,9,12	
			<i>MOM2</i>	1,3,8,9,12	
			<i>LTA</i>	2,3,10,15,16	
			<i>SDHB</i>	1,3,9,10,16	
			<i>POLE2</i>	1,3,8,9,17	
			<i>COX7C</i>	1,9,10,12,16	
			<i>COX8A</i>	1,9,10,12,16	
	<i>NDUFB7</i>		1,9,10,12,16		
	<i>NDUFA8</i>		1,9,10,12,16		
	<i>NDUFA7</i>		1,9,10,12,16		
	<i>SSBP1</i>		1,8,9,12,17		
	Reactome	<i>NUP93</i>	1,3,9,10,11,12,17		
		<i>POLE2</i>	1,3,9,11,16,17		
		<i>POLR2L</i>	1,3,9,11,12,16		
		<i>POLR2G</i>	1,3,9,12,16,17		
		<i>PSMD14</i>	1,3,9,11,12,17		
		<i>PSMC6</i>	1,3,9,11,12,17		
		<i>RPA2</i>	1,3,9,11,12,17		
		<i>NUP85</i>	1,3,9,10,11,12		
		<i>NUP37</i>	1,3,9,11,12,17		
		<i>NUP205</i>	1,3,9,11,12,17		
	Gene Ontology	n/a	n/a		
		BioCarta	n/a	n/a	
			KEGG	<i>EP300</i>	1,3,6,12,13
				<i>ACTN1</i>	1,6,12,13,14
				<i>PLOB2</i>	1,3,8,13
				<i>PIK3OG</i>	1,6,8,13
				<i>CREBBP</i>	1,3,12,13
	<i>MAPK9</i>	1,2,6,13			
	<i>LEF1</i>	1,3,6,12			
	<i>PIK3R3</i>	2,6,8,13			
<i>AKR104</i>	3,8,16,17				

Table 4. Cont.

Up-regulated in	Gene Sets database	Gene	Shared by twin pair ID
		<i>PARD3</i>	6,8,12,16
	Reactome	<i>MNAT1</i>	2,6,7,14
		<i>RFC2</i>	2,6,7,14
		<i>PRIM1</i>	2,6,7,14
		<i>PRIM2</i>	2,6,7,14
	Gene Ontology	<i>CHRNA2</i>	1,9,16,17
		<i>KCNE2</i>	1,9,16,17
		<i>KCNQ3</i>	1,9,16,17
		<i>INHBA</i>	1,9,14,17
		<i>SLC34A3</i>	1,5,16,17

n/a, no gene was shared by at least 4 twin pairs.
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demonstrated improved performance in various tests of learning and memory [39].

Identified by utilizing BioCarta pathway database, *IGF1* manifested up-regulation in higher IQ twins. The insulin-like growth factor (IGF) system is important in growth and development. While the exact mechanism remains unknown, the growth hormone (GH) and IGF-1 axis has been reported to play a role in the reduction of cognitive functions in aging population and patients with GH deficiency [20]. Methylation status of its promoter regions was studied, yet no difference was discerned between the twins. It is possible that *IGF1* is under some other epigenetic regulation, considering the actual mechanisms responsible for the cell type-specific expression patterns of this gene remain to be elucidated [40].

Since intelligence is a complex trait associated with many genes of small effect [41,42], it was not surprising that we failed to identify a single gene manifesting prevailing expression changes across all 17 twin pairs. We also noticed that by microarrays none of the candidate genes identified by methylation analyses was listed in the results of expression studies. A reason of the discrepancy of these two methods may be the lack of comprehensive accession to all known epigenetic regulations. DNA methylation at CpG sites across promoter regions has been deeply studied, while a number of other epigenetic regulatory mechanisms are also found to modulate gene expression [22]. Furthermore, ever since the genome-wide, single-base human DNA methylome mapping became possible, the correlation of methylation status of gene bodies and expression levels has been gaining attention [43]. It has been documented that DNA methylation of gene bodies is associated with gene activity. Therefore, it is possible that the intelligence-related expression profiles were subjected to this novel epigenetic regulation.

The present study has its conceptual and technical limitations. Conceptually, we hypothesized that changes in methylation status and expression levels could be captured in genomic DNA and total RNA extracted from whole blood and derivative lymphoblastic cell lines, respectively. Given the inability to probe DNA methylation status or gene expression in the human brain, except in postmortem studies, human blood is commonly used in transcriptional studies of various diseases including psychiatric disorders [44]. Although there is still no consensus regarding blood-based gene expression profiles as good surrogates for addressing neuroscientific research, the moderate correlation

between transcripts in whole blood and the central nervous system makes it an accessible alternative [45,46]. Several studies utilizing similar strategies to study twin pairs discordant for psychiatric disorders through comparisons of CpG islands methylation of peripheral blood cells or lymphoblast cell lines had detected a number of disease-associated epigenetic changes [47–49]. Moreover, it has been documented that the methylation changes of large-scale domains are linked to cell-specific differentiation [50]. Several functional gene sets we found (i.e., “cation channel activity”, “cell-cell signaling”, “extracellular region part”, “G-protein coupled receptor protein signaling pathway”, and “gated channel activity”) were observed within neuronal highly methylated domains. The association between expression difference observed in lymphoblast cell lines and neuron-specific methylation patterns implies that these candidate gene sets are more likely to reflect the true differences in brains. Technical limitations of this study include low fold-change differences in expression levels manifested between co-twins. We did not carry out qRT-PCR for the genes identified by GSEA, considering that differences of less than 1.5-fold are thought to be beyond the limit of reproducibility [51].

Reverse causality should be considered in epigenetic studies, considering all known epigenetic marks are influenced by environmental exposures including diet, smoking, alcohol consumption, stress, or physical activities [52]. It might be plausible that the changes we observed in this study resulted from the divergent lifestyle choices by subjects with different levels of intelligence, and not that these epigenetic changes caused the twins to differ.

Here, we presented the first study that used genome-wide epigenetic and transcriptomic profiling to identify epigenetic changes related to the discordance between MZ twins with normal-range intelligence. A list of new candidate genes possibly related to cognitive abilities was generated while further replications and functional analysis remain necessary.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Attendance was voluntary, and signed informed consent including information on genetic analyses was obtained from all participants. The Ethical Committees of Kobe University Graduate School of Medicine and Keio University Faculty of Letters approved study protocols.

Samples

A sub-sample of 326 twin pairs of twins from the Keio Twin Project were invited to Keio University, where the Kyodai Nx15-, one of the most often used group intelligence tests in Japan, was applied. The zygosity of participants was diagnosed by 15 polymorphic STR loci (AmpF Φ STR identifier kit, Applied Biosystems). Among the 240 pairs to have monozygosity, 34 MZ twin pairs who manifested differences in IQ score of more than 15 points between co-twins. One pair was excluded for a lower-than-normal IQ score (52 points). From the 17 of the remaining 33 twin pairs, who agreed to participate in the study, peripheral blood was drawn and B-lymphoblastoid cell lines were established. For the treatment of 5-azadC (WAKO), a daily aliquot of 5 mM stock solution was added to flasks and thoroughly resuspended (final concentration of 1 μ M). Cells were harvested after 3 days from the start of treatment.

Nucleic acids extraction

For human promoter microarrays and bisulfite genomic sequencing, genomic DNA was extracted from the blood via established methods. For gene expression microarrays and quantitative RT-PCR, total RNA was isolated using RNeasy Plus Mini kit (QIAGEN) from B-lymphoblastoid cell lines.

DNA methylation profiling

One microgram of genomic DNA was sonicated and subjected to methylated DNA enrichment using the MethylMiner methylated DNA enrichment kit (Invitrogen) as per the manufacturer’s instructions. The methylated DNA fragments, amplified by the GenomePlex WGA reamplification kit 3 (SIGMA) and supplemented with dUTP, were further purified using the QIAquick PCR purification kit (QIAGEN).

According to Affymetrix’s chromatin immunoprecipitation assay protocol, enriched methylated DNA was hybridized to GeneChip Human Promoter 1.0R arrays (Affymetrix), which comprised a coverage of over 25,500 human promoter regions.

AGCC (Affymetrix GeneChip Command Console)-format CEL files were first created, and then converted to GCOS (GeneChip Operating Software, Affymetrix)-format CEL files. For the pairwise analyses, paired CEL files were imported into MAT software to specify candidate regions (approximately 600 base pairs in length) with significantly different probe intensities between co-twins ($p < 10^{-6}$).

To detect candidate loci across all 17 twin pairs, we utilized Partek Genomic Suite 6.5 software (Partek) to import the CEL files, and have the data converted to \log_2 values after normalized by the RMA (Robust Multichip Averaging) algorithm. After the signal from each probe for the higher-IQ sibling was subtracted from that of the lower-IQ co-twin across all probes, one-class *t*-test with statistical parameters set at $p < 10^{-6}$ was carried out to detect significant regions.

Bisulfite sequencing

Genomic DNA was bisulfite-treated using the Methylcode bisulfite conversion kit (Invitrogen) as per the manufacturer’s instructions. Amplification was performed with Takara LA Taq polymerase, with converted DNA-specific primers that were designed using MethPrimer. The amplicons were cloned into vectors using a TOPO TA cloning kit (Invitrogen). We performed direct sequencing of the plasmid DNA that was isolated using PI-200 auto-plasmid-isolator (KURABO) via the ABI 3730xl sequencing system (Applied Biosystems).

Quantitative RT-PCR

Two micrograms of the total RNA was subjected to reverse transcription using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Gene target amplifications, using Takara SYBR premix Ex Taq, were performed in triplicate in a matter of a serial 10 fold dilution. Housekeeping gene *GAPDH* served as the internal control gene. Mann-Whitney U test was performed to compare the relative expression levels between co-twins.

Gene expression profiling

We processed 300 nanograms of total RNA using the Ambion WT expression kit and the Affymetrix GeneChip WT terminal labeling kit according to the manufacturers’ recommended methods. Hybridization and scanning of GeneChip Human Gene 1.0 ST arrays (Affymetrix), which comprised of more than 28,000 gene-level probe sets, were performed as per the manufacturer’s

instructions. Partek Genomic Suite 6.5 software was used to import AGCC-format CEL files and normalize the data according to the RMA algorithm. ANOVA with an FDR-adjusted p set to 0.05 was used to determine those probe sets that were significantly different between the groups of twins with a higher IQ and their lower IQ co-twins. A one-class t -test analysis with multiple sample correction was conducted across all \log_2 ratios (higher-IQ twin/lower-IQ co-twin) for all 17 twin pairs. We also carried out pairwise comparison for the expression array data and then included genes with a fold-change value more than 2. Genes replicated in the same tendency (up-regulated in the higher IQ twins, or up-regulated in the lower IQ twins) in most pairs were listed.

In another approach, the twins of each pair were categorized into higher and lower expression groups according to the expression level of every individual gene. A paired t -test was carried out to compare the mean IQ scores of the two groups. Corrected p of 10^{-6} was applied as the cutoff to define being positive.

GSEA was performed for functionally related genes across a spectrum of gene sets of C2 curated gene sets including BioCarta gene sets (217 gene sets), KEGG gene sets (186 gene sets), and Reactome gene sets (430 gene sets), and C5 GO gene sets (1,454 gene sets) separately. Pre-ranked gene lists, including lists with up-regulated/down-regulated genes in the group of twins with higher IQ scores sorted according to the p calculated by a between-group ANOVA test, and lists for each twin pair with genes sorted by between-sibling fold-change values, were constructed for the analyses. Gene sets with FDR q -value < 0.25 after 1,000 permutation cycles were considered significantly enriched. Lists of leading edge subset genes, the cores of gene sets that account for the enrichment signal, were then generated. To create the list of enriched genes shared by plural twin pairs, the upper 100 enriched genes of each pair were included in the test.

Supporting Information

Dataset S1 Pair-wise comparison of expression array data. Genes with a fold-change value >2 were included. The positive value of fold-change designates up-regulation in the higher IQ twin, while the negative value designates the other way round. n/a indicates no gene matched the fold-change cutoff value. (XLS)

Dataset S2 Pair-wise GSEA results using BioCarta database. Gene sets with a FDR q -value < 0.25 were included. n/a indicates no gene set matched the FDR cutoff value. (XLS)

Dataset S3 Pair-wise GSEA results using KEGG pathway database. Gene sets with a FDR q -value < 0.25 were included. n/a indicates no gene set matched the FDR cutoff value. (XLS)

Dataset S4 Pair-wise GSEA results using Reactome database. Gene sets with a FDR q -value < 0.25 were included. n/a indicates no gene set matched the FDR cutoff value. (XLS)

Dataset S5 Pair-wise GSEA results using GO database. Gene sets with a FDR q -value < 0.25 were included. n/a indicates no gene set matched the FDR cutoff value. (XLS)

Figure S1 Scatterplot of the 240 MZ twin pairs IQ scores. This diagram provides an overview of the IQ distribution for all 240 MZ twins from the Keio Twin Study. Each circle identifies one twin pair with its x-coordinate and y-coordinate

representing, respectively, the IQ score of Twin A and Twin B. With the black line standing for regression, the correlation coefficient of 0.72 suggests the similarities between twins. Circles located outside of the space between two blue lines indicate twin pairs manifesting between-sibling IQ differences larger than 15 points and were considered to be recruited, while the red arrowhead points to one pair being excluded as a possible subject for a lower-than-normal IQ score.

(TIF)

Figure S2 Positive correlation between IQ scores differences and the number of loci different in methylation status. The positive correlation between the number of loci with significant differences in methylation patterns and the differences of IQ scores of the twin pairs was visualized. Twin pairs with larger differences in IQ scores tended to have more loci identified by screening for epigenetically regulated genes.

(TIF)

Figure S3 PCA results for the expression profiles of 17 twin pairs. Principle components ranked from the highest variance are named accordingly as PC 1st and PC 2nd. The PCA projection maps these two components data to 2 dimensions for visualization. In the scatter plots, each point represents a sample. The color of the symbol represents the relative IQ scores with red as higher and blue as lower. The number on each symbol indicates the twin pair ID. In contrast to the similarity between co-twins from each pair, no apparent gathering pattern could be recognized by the relative IQ scores.

(TIF)

Figure S4 Clustering analysis for the expression profiles of 17 twin pairs. Clustering analysis for the list of 644 genes manifesting more than a 1.1-fold change between the groups of twins with higher IQ scores and the groups of their co-twins under ANOVA analysis was performed. The number below each symbol indicates the twin pair ID. The color red and blue of the symbol indicate respectively the higher IQ and the lower IQ twin of each twin pair. The scale represents fold changes, according to the color map at the bottom of the figure. No apparent clustering could be recognized.

(TIF)

Table S1 Summary of candidate loci with methylation changes identified by promoter DNA methylation patterns and their bisulfite sequencing result.

(DOC)

Table S2 Up-regulated gene sets in the group of twins identified by GSEA (FDR q -value < 0.25).

(DOC)

Table S3 Methylation profiling by bisulfite sequencing for *IGF1* (Chr2:101335584–131398508) in Twin Pair ID 7.

(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CCY MF KK TT. Performed the experiments: CCY MF. Analyzed the data: CCY MF. Contributed reagents/materials/analysis tools: CS PCC JS HS KI TK JA. Wrote the paper: CCY MF KK TT.

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SHORT REPORT

A multi-centre clinico-genetic analysis of the VPS35 gene in Parkinson disease indicates reduced penetrance for disease-associated variants

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ABSTRACT

Background Two recent studies identified a mutation (p.Asp620Asn) in the vacuolar protein sorting 35 gene as a cause for an autosomal dominant form of Parkinson disease. Although additional missense variants were described, their pathogenic role yet remains inconclusive. **Methods and results** We performed the largest multi-center study to ascertain the frequency and pathogenicity of the reported vacuolar protein sorting 35 gene variants in more than 15,000 individuals worldwide. p.Asp620Asn was detected in 5 familial and 2 sporadic PD cases and not in healthy controls, p.Leu774Met in 6 cases and 1 control, p.Gly51Ser in 3 cases and 2 controls. Overall analyses did not reveal any significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort.

Conclusions Our study apart from identifying the p.Asp620Asn variant in familial cases also identified it in idiopathic Parkinson disease cases, and thus provides genetic evidence for a role of p.Asp620Asn in Parkinson disease in different populations worldwide.

INTRODUCTION

There is increasing interest to try to identify uncommon and rare genetic variants that increase the risk of common diseases and that are difficult to identify using traditional genome-wide association studies (GWAS) approaches.¹ Rare variants which are not mapped by GWAS can be identified by using next generation sequencing, that is, exome sequencing in large families with multiple affected individuals.² Exome sequencing is now

routinely used to identify rare mutations in familial forms of disease in diverse phenotypes.²

Two recent studies independently performed exome sequencing in large families of Caucasian descent, and identified a mutation in the vacuolar protein sorting 35 (VPS35) gene as a possible cause for an autosomal dominant form of Parkinson disease (PD).^{3,4} In addition, several non-synonymous base exchanges were identified, but their involvement in disease pathogenesis remains inconclusive. Furthermore, recently published studies provided conflicting results regarding the role of VPS35 in PD.⁵⁻⁸ Here, we performed a large multi-centre study to determine the frequency and pathogenicity of VPS35 variants in PD in diverse populations worldwide.

METHODS

Consortium

Investigators from the Genetic Epidemiology of Parkinson disease Consortium were invited to participate in this study. A total of 23 sites representing 19 countries from four continents agreed to contribute DNA samples and clinical data for a total of 15 383 individuals (8870 cases and 6513 controls). Control individuals underwent neurological examination and were excluded from the study whenever there was clinical evidence for any extrapyramidal disorder.

Genotyping

We selected seven non-synonymous variants exactly as they were proposed.³ In addition, we selected tag single nucleotide polymorphisms

(SNPs) (HapMap Rel 28 phase II+III, Aug10, National Centre for Biotechnology Information. B36 dbSNP b126; <http://www.hapmap.org>) that cover the common genetic variants in the VPS35 gene using an r^2 threshold of 0.8–1.0 to select tag SNPs for VPS35 gene. Using this strategy, we were able to capture 23 SNPs in a 40 kb region, including VPS35 ('chr16:46 693 589–46 723 144 based on hg 19'). Therefore, in total, 10 SNPs located in the VPS35 were genotyped (including seven rare non-synonymous and three common variants). Genotyping was performed by a central genotyping core. Genotyping was performed using a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry on a MassArray system (Sequenom, San Diego, California, USA). Cleaned extension products were analysed by a mass spectrometer (Bruker Daltonic, Billerica, MA, USA) and peaks were identified using the MassArray Typer 4.0.2.5 software (Sequenom). Assays were designed by the AssayDesigner software 4.0 (Sequenom) with the default parameters for the iPLEX Gold chemistry and the Human Genotyping Tools ProxSNP and PreXTEND (Sequenom). All variants were genotyped in one multiplex assay. The average call rate of the variants was >97%. The local Ethics Committee approved the study. All participants gave signed informed consent.

Statistical analysis

Logistic regression was used to test the association between VPS35 and PD in our overall cohort. For common variants (minor allele frequency >5%), we synthesised the effect estimates using fixed and random effects models. Fixed effect models assume that the genetic effect is the same in populations from different sites and that observed differences are due to chance alone. For associations showing between-study heterogeneity, fixed effect estimates yield narrower CIs and smaller *p* values as compared with random effects models, which incorporate between-study heterogeneity.^{9–10} Random effects models allow the genetic effects might be different due to genuine heterogeneity that may exist across different sites. Random effects calculations take into account the estimated between-study heterogeneity. Cochran's Q test of homogeneity and the I^2 metric were used to evaluate the between-site heterogeneity. The I^2 metric ranges from 0% to 100% and measures the proportion of variability that is beyond chance. Typically, estimates of I^2 <25% are considered to reflect little or no heterogeneity, 25%–50% moderate heterogeneity, 50%–75% large heterogeneity and >75% very large heterogeneity. The overall main analysis considered all sites and populations irrespective of ancestry. For variants with minor allele frequency <1%, an exact test was used to compare the frequency differences between cases and controls combining data across all 21 sites.

RESULTS

Characteristics of sites and overall database

Overall, 23 sites contributed a total of 8870 cases and 6513 controls. Characteristics of all participating sites are shown in table 1. Most sites contributed participants of Caucasian ancestry (N=19); four sites included participants of Asian ancestry. The proportion of men and women ranged from 42% to 58% across different participating sites (table 1). The median age at onset of PD in our studied population was 61 years.

Rare variants

Overall, we observed p.Asp620Asn in seven cases, p.Leu774Met in six cases and one control, p.Gly51Ser in three cases and two

controls. Details per site are shown in table 2. The controls subjects carrying p.Leu774Met (P-13) and p.Gly51Ser (P-2 and P-16) at the time of study sampling were 81, 84 and 76 years, respectively. In Caucasian populations, the number of carriers in cases and controls for the three variants were 5 versus 0 (p.Asp620Asn), 4 versus 1 (p.Leu774Met) and 3 versus 1 (p.Gly51Ser), respectively. In Asian descent populations, the respective numbers were 2 versus 0 (p.Asp620Asn), 2 versus 0 (p.Leu774Met) and 0 versus 1 (p.Gly51Ser). Most interestingly, two out of seven patients carrying the p.Asp620Asn variant presented without any family history for PD. This represents the first evidence for reduced penetrance of the respective variant initially attributed to autosomal dominant familial PD. We did not observe any carriers for one variant (p.Arg524Trp) in our cohort. Two non-synonymous variants (p.Met57Ile, p.Thr82Arg) failed genotyping. By collapsing the rare variants across different sites, we did not observe statistically significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort (see online supplementary table S1).

Overall data synthesis for common variants

Out of three tag SNPs, one SNP (rs3218745) failed genotyping. We did not observe significant association for any of common variants with PD either with either fixed effect or random effect models (see online supplementary table S2). The OR ranged from 0.96 to 0.99 and tight 95% CIs excluded modest association effects. We observed no substantial heterogeneity for the two genotyped SNPs, and also the Q test was non-statistically significant for common SNPs. Moreover, examining the Caucasian or Asian populations separately did not change our results (data not shown).

Clinical features

All PD patients who carried potential pathogenic variants (p.Asp620Asn, p.Gly51Ser, p.Leu774Met) were clinically diagnosed with PD (Online supplementary clinical analysis data). A few of these (0.2%) affected individuals also have a positive family history. Affected individuals exhibited classical symptoms of PD (resting tremor, bradykinesia, rigidity) (table 2). The clinical diagnosis of PD was made by movement disorder specialists who used UK brain bank criteria for PD. Non-motor symptoms were present in the majority of PD patients carrying a pathogenic variant (table 2). Interestingly, hallucinations and dementia were also observed in one asymptomatic carrier suggesting clinical heterogeneity associated with VPS35. The identified healthy carriers have not shown any sign of PD as yet (table 2).

DISCUSSION

We performed the first multi-centre study to define the role of the VPS35 gene (PARK17) in PD by assessing the frequency of the reported non-synonymous variants in familial and sporadic PD patients from different populations worldwide. Among 15 383 subjects genotyped, we found a pathogenic relevance for p.Asp620Asn in different populations. Most interestingly, out of seven subjects who carry p.Asp620Asn, two have a negative family history. Therefore, our results provide additional evidence that VPS35 is a rare cause of familial as well as the common sporadic form of PD. In total, about 0.4% of PD cases in diverse population were due to disease-associated variant in the VPS35 gene. Our lack of supporting the role of common variants of the VPS35 gene in PD is consistent with recently published GWAS and also meta-analyses of GWAS of PD, as none of these highlighted the role of common variability in VPS35 gene as a risk factor for PD.^{11–15} The p.Asp620Asn

Table 1 Description of datasets contributed by each study site

Site	Country	N	Case	Control	Male (%)	Female (%)	Mean AAO	Mean Age at study	Diagnostic criteria
Annesi	Italy	394	197	197	204 (51.7%)	190 (48.2%)	61.5	63.7	UKPDBB
Brice	France	505	272	233	302 (59.8%)	203 (40.1%)	47.6	57.8	UKPDBB
Bozi	Greece	222	114	108	107 (48.1%)	115 (51.8%)	69.9	74.5	UKPDBB
Wszolek	USA	1518	692	826	794 (52.3%)	724 (47.6%)	64.4	71.7	UKPDBB
Garraux	Belgium	82	68	14	45 (54.8)	37 (45.1%)	62.1	69.6	UKPDBB
Hadjigeorgiou	Greece	714	357	357	379 (53.0%)	335 (46.9%)	63.4	63.7	UKPDBB
Jeon	Korea	749	408	341	314 (41.9%)	435 (58.0%)	57.6	NA	UKPDBB
Opala	Poland	629	352	277	340 (54.0%)	288 (45.7%)	50.2	68.1	UKPDBB
Lynch	Ireland	740	368	372	340 (45.9%)	400 (54.0%)	50.5	70.7	UKPDBB
Lin	Taiwan	320	160	160	160 (50%)	160 (50%)	62.0	70.8	UKPDBB
Facheris	Italy	181	114	67	86 (47.5%)	95 (52.4%)	63.0	NA	UKPDBB
Maraganore	USA	1024	801	223	600 (58.5%)	361 (35.3%)	59	74.7	Bower
Mellick	Australia	2024	1012	1012	1042 (51.4%)	981 (48.4%)	59	72.2	Bower
Morrison	England	1120	766	354	606 (54.1%)	514 (45.8%)	66.1	NA	UKPDBB
Mok	China	436	260	176	264 (60.5%)	170 (38.9%)	NA	63.5	UKPDBB
Aasly	Norway	1278	656	622	721 (56.4%)	557 (43.5%)	58.8	72.9	UKPDBB
Wirdefeldt	Sweden	299	83	216	147 (49.1%)	152 (50.8%)	65.8	71.4	Gelb
Van Broeckhoven	Belgium	1010	501	509	500 (49.5%)	509 (50.3%)	60.5	66.3	Pals/Gelb
Rogaeva	Canada	560	387	173	303 (54.1%)	257 (45.8%)	49.7	64.2	UKPDBB
Tan	Singapore	391	194	197	244 (62.4%)	147 (37.5%)	59.7	54.0	UKPDBB
Hattori	Japan	121	121	0	62 (51.2%)	59 (48.7%)	NA	NA	UKPDBB
Gasser/Sharma	Germany	760	760	0	479 (63.3%)	281 (36.9%)	58.9	NA	UKPDBB
Toda	Japan	306	227	79	161 (52.6%)	145 (47.3%)	57.8	65.1	UKPDBB
Total		15 383	8870	6513			59.5	67.6	

AAO, Age at onset; NA: Not applicable.

variant is located in the C-terminal region of the VPS35 protein pointing that subtle structural changes might influence the disease pathogenesis.³

The spectrum of proteins involved in PD aetiology has grown considerably. This includes proteins that are related to mitochondrial quality control (Parkin, PINK1 and DJ1), proteins involved in protein aggregation (SNCA) (Synuclein, MAPT) Microtubule associated protein Tau), and proteins

which are involved in sorting and degradation within endocytic and autophagy pathways ((VDAC) Voltage dependent anion channel, (GBA) Glucocerebrosidase gene, VPS35).^{16 17} So far, very little is known about the specific role of VPS35 in PD, except that it is hypothesised that it is involved in cargo recognition as part of a retrograde complex recycling membrane proteins from endosomes to the trans-Golgi network.^{3 4} Indeed, in vitro and in vivo studies strongly implicate the role of VPS35

Table 2 Clinical description of carriers of non-synonymous variants of vacuolar protein sorting 35 gene

Id	Ethnicity	Rare variant	Age at onset	Clinical signs	Bradykinesia	Rigidity	Tremor	Postural instability	L-dopa responsive	Non-motor symptoms	Family history
P-1	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Negative
P-2	Caucasian	p.Gly51Ser	NA	Control	-	-	-	-	-	Negative	Negative
P-3	Caucasian	p.Gly51Ser	NAV	Classical PD	+	+	+	+	+	Dementia, visual hallucinations	Negative
P-4	Caucasian	p.Gly51Ser	55	Classical PD	+	+	+	+	+	Negative	Negative
P-5	Caucasian	p.Gly51Ser	49	Classical PD	+	+	+	+	+	Negative	Negative
P-6	Caucasian	p.Asp620Asn	37	Classical PD	+	+	+	+	+	Negative	Positive
P-7	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Positive
P-8	Caucasian	p.Asp620Asn	55	Classical PD	+	+	+	+	+	Negative	Positive
P-9	Caucasian	p.Asp620Asn	66	Classical PD	+	+	+	+	+	Negative	Positive
P-10	Caucasian	p.Leu774Met	41	Classical PD	+	+	+	+	+	Negative	Positive
P-11	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Negative	Positive
P-12	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Disturbance of gait and balance	Positive
P-13	Caucasian	p.Leu774Met	NA	Control	-	-	-	-	NA	-	Negative
P-14	Caucasian	p.Leu774Met	44	Classical PD	+	+	+ Rest 1st sx	+	+	Autonomic dysfunction	Positive
P-15	Asian	p.Asp620Asn, p.Leu774Met	52	Classical PD	+	+	+	+	+	Negative	Negative
P-16	Asian	p.Gly51Ser		Control	-	-	-	-	-	-	Negative
P-17	Asian	p.Leu774Met	75	Classical PD	+	+	+	+	+	Negative	Negative
P-18	Asian	p.Asp620Asn	43	Classical PD	+	+	+	+	+	Mild cognitive impairment	Positive

NA, not applicable; NAV, not available; PD, Parkinson disease; + positive; - negative.

gene in neurodegeneration. For example, reduced levels of VPS35 have been found in affected brain regions of Alzheimer disease (AD) patients¹⁸ and loss of VPS35 function has been shown to increase the levels of amyloid β and cause synaptic impairment in a mouse model of AD. Furthermore, variants in another member of the VPS family and substrate of retromer complex, SORL1, have been implicated in AD.¹⁹

In this study, we have focused only on non-synonymous variants identified by Zimprich and colleagues.³ Of note, we confirmed the pathogenic relevance of the p.Asp620Asn variant which was identified by both studies for familial cases and in sporadic PD. Recently, published studies also identified p.Asp620Asn mutations in PD,^{6, 20} thus providing support to the role of p.Asp620Asn in PD. In our study, clinically, the symptomatic carriers showed a broad spectrum of clinical phenotypes ranging from typical PD to (DLB) Dementia with Lewy body, so longitudinal evaluation of carriers at risk will provide unique information on the natural course of the disease caused by VPS35. Even though our data support the role of p.Asp620Asn variant in PD, given the fact that the frequency in diverse population is far below <1%, it is likely to be a rare cause of PD worldwide. Nevertheless, sequencing of families is encouraged for identifying additional missense variants which may provide mechanistic insight into the causes of PD.

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Hsp40 Gene Therapy Exerts Therapeutic Effects on Polyglutamine Disease Mice via a Non-Cell Autonomous Mechanism

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Abstract

The polyglutamine (polyQ) diseases such as Huntington's disease (HD), are neurodegenerative diseases caused by proteins with an expanded polyQ stretch, which misfold and aggregate, and eventually accumulate as inclusion bodies within neurons. Molecules that inhibit polyQ protein misfolding/aggregate, such as Polyglutamine Binding Peptide 1 (QBP1) and molecular chaperones, have been shown to exert therapeutic effects *in vivo* by crossing of transgenic animals. Towards developing a therapy using these aggregation inhibitors, we here investigated the effect of viral vector-mediated gene therapy using QBP1 and molecular chaperones on polyQ disease model mice. We found that injection of adeno-associated virus type 5 (AAV5) expressing QBP1 or Hsp40 into the striatum both dramatically suppresses inclusion body formation in the HD mouse R6/2. AAV5-Hsp40 injection also ameliorated the motor impairment and extended the lifespan of R6/2 mice. Unexpectedly, we found even in virus non-infected cells that AAV5-Hsp40 appreciably suppresses inclusion body formation, suggesting a non-cell autonomous therapeutic effect. We further show that Hsp40 inhibits secretion of the polyQ protein from cultured cells, implying that it inhibits the recently suggested cell-cell transmission of the polyQ protein. Our results demonstrate for the first time the therapeutic effect of Hsp40 gene therapy on the neurological phenotypes of polyQ disease mice.

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Introduction

The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders that are all caused by a common genetic mutation, namely an expansion (>40) of a polyQ-encoding CAG repeat in each unrelated disease-causing gene [1,2]. Nine polyQ diseases have been identified to date, including Huntington's disease (HD), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, 17, dentatorubral pallidolusian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA). In these disorders, progressive degeneration of neurons in brain areas specific for each disorder occurs, causing various neurological and psychiatric symptoms corresponding to each affected brain area [1,2].

In the common molecular pathogenesis of the polyQ diseases, proteins with an expanded polyQ stretch become misfolded and form aggregates, and subsequently accumulate as inclusion bodies within neurons, eventually resulting in neurodegeneration [3–7]. Moreover, recent studies suggest that prion-like transmission of

aggregation-prone proteins between cells is involved in neuro-pathological spreading during disease progression in not only the polyQ diseases but also various other neurodegenerative diseases [8–11]. Although various therapeutic strategies against downstream targets of the pathogenic cascade have been investigated, misfolding and aggregation of the polyQ protein are ideal therapeutic targets since they are the most initial pathogenic events, and therefore their inhibition is expected to result in the suppression of a broad range of downstream pathogenic events [3,5,12,13].

In our attempt to establish a therapy for the polyQ diseases, we hypothesized that molecules that specifically bind to the expanded polyQ stretch would suppress misfolding and aggregation of the expanded polyQ protein. Accordingly, by phage display screening of combinatorial peptide libraries, we identified PolyQ Binding Peptide 1 (QBP1), and proved that QBP1 indeed inhibits polyQ protein misfolding/aggregation *in vitro* [14–16]. Furthermore, we

demonstrated that expression of QBP1 suppresses neurodegeneration *in vivo* in polyQ disease model animals [16,17]. Another approach for targeting misfolding and aggregation of the expanded polyQ protein is to utilize molecular chaperones, which are a group of biomolecules that assist the proper folding of proteins and prevent protein misfolding/aggregation [18–20]. Indeed, overexpression of molecular chaperones such as Hsp40 and Hsp70 has been shown to suppress polyQ protein aggregation and polyQ-induced neurodegeneration in various animal models of the polyQ diseases, such as flies [21–24], worms [25] and mice [26–30]. However, most studies showing the therapeutic efficacy of these aggregation inhibitors have been performed by crossing transgenic animals so far. To develop a therapy using aggregation inhibitors such as QBP1 and molecular chaperones, transgenes need to be delivered in affected individuals by administration, rather than be expressed in the next generation by crossing transgenic animals.

In this study, we employed a viral vector to deliver these transgenes into the brain and investigated their therapeutic effects on polyQ disease model mice. Among various viral vectors, we chose to use adeno-associated virus vector (AAV) because of its widespread infection throughout the brain, its long-term expression of transgenes, and its safety [31,32]. We successfully demonstrate the therapeutic effects of AAV5-QBP1 and AAV5-Hsp40 injections on a mouse model of HD. Most interestingly, we found that AAV5-Hsp40 exerts a non-cell autonomous therapeutic effect, possibly via inhibition of the recently-suggested cell-cell transmission of the polyQ protein, indicating a novel therapeutic mode of action of Hsp40.

Results

AAV5-QBP1 and AAV5-Hsp40 Inhibit Inclusion Body Formation in polyQ Disease Mouse Neurons

We employed the R6/2 HD mouse model [33] to investigate the therapeutic effect of AAV-mediated expression of QBP1 and molecular chaperones. We first tested the effect on accumulation of the pathogenic polyQ protein into inclusion bodies in the neurons of R6/2 mice by AAV5 injections. Injections were performed on mice at postnatal day 7 (P7) using an infusion pump (see Materials and Methods), which has been shown to lead to widespread delivery of the injected molecules in the brain [34], and indeed resulted in widespread expression of the transgene throughout the injected brain hemisphere with ~30% infection efficiencies (Fig. S1). R6/2 mice were injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 on the other side, and htt inclusion body formation was compared between the two sides of both the striatum and the cortex. Inclusion bodies were already formed in 36.0% of AAV5-GFP infected neurons in the striatum at 4 weeks of age, which increased to 68.5% at 8 weeks and 73.8% at 14 weeks, and an age dependent increase in inclusion bodies was also observed in the cortex (Fig. S2). In contrast, AAV5-QBP1 infected neurons had significantly less inclusion bodies at most time points (Fig. S2), and the rates of neurons with inclusion bodies at 8 weeks, for example, were 68.5% for GFP vs 33.7% for QBP1 ($p < 0.001$) in the striatum, and 49.3% for GFP vs 27.4% for QBP1 ($p < 0.001$) in the cortex (Figs. 1A,B). These results demonstrate a significant inhibitory effect of AAV5-QBP1 on inclusion body formation.

We also tested the effect of AAV5-mediated expression of a molecular chaperone on inclusion body formation. Among the various molecular chaperones, we chose to use a member of the Hsp40 family, namely DNAJB1 (referred to as Hsp40 in this study), since members of the DNAJB subfamily have been

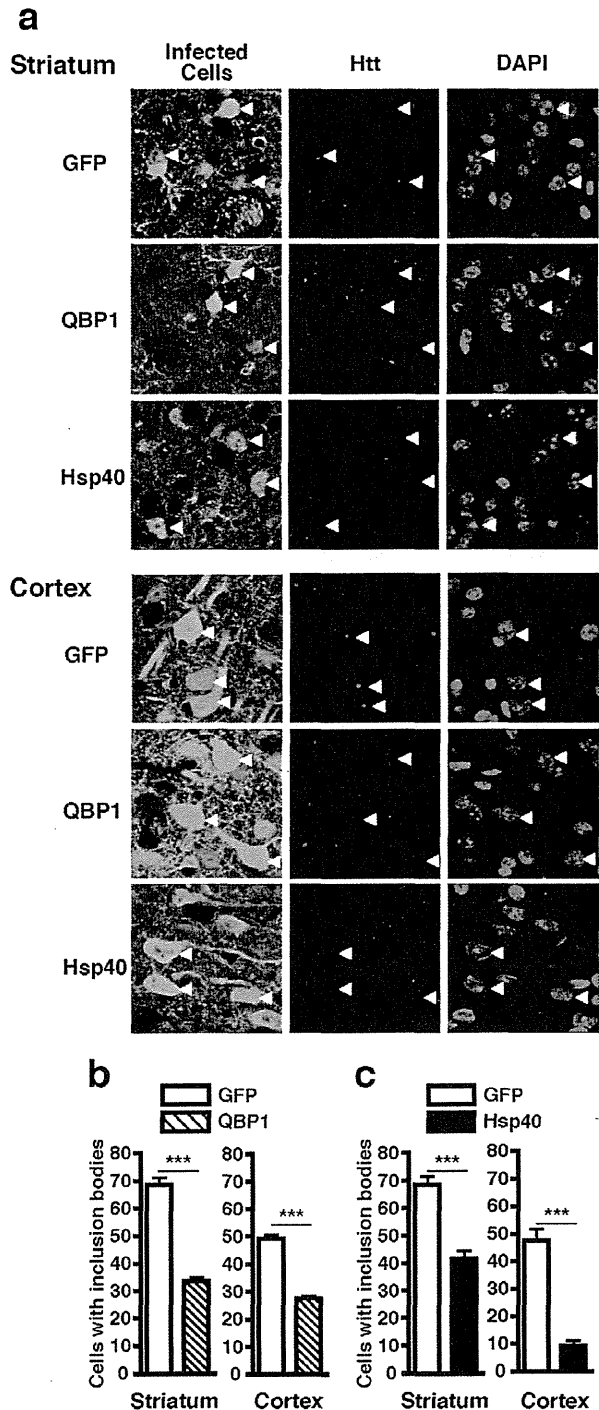


Figure 1. AAV5-QBP1 and AAV5-Hsp40 inhibit polyQ inclusion body formation in virus infected neurons of polyQ disease mice. R6/2 mice at P7 were injected with AAV5-GFP on one side of the striatum and either AAV5-QBP1 or AAV5-Hsp40 on the other side, and htt inclusion body formation in virus infected neurons was assessed at 8 weeks of age by immunohistochemistry of brain sections. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice, injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. In each panel, representative virus infected cells are indicated by white

arrowheads. (B) Inclusion body formation in AAV5-QBP1 infected neurons in the striatum (left) and cortex (right). (C) Inclusion body formation in AAV5-Hsp40 infected neurons in the striatum (left) and cortex (right). In (B) and (C), data are shown as means \pm SEM of ≥ 6 fields of view, in which over 180 cells were counted ($*p < 0.05$, $***p < 0.001$). Representative results of two mice analyzed are shown. doi:10.1371/journal.pone.0051069.g001

reported to be the most potent suppressors of expanded polyQ protein aggregation and toxicity [35]. The effect of AAV5-Hsp40 on polyQ inclusion body formation in R6/2 mice was investigated at 8 weeks, an age at which AAV5-QBP1 showed a clear inhibitory effect. AAV5-Hsp40 also exerted a robust effect on inclusion body formation, and the rates of virus infected neurons with inclusion bodies were 68.6% for GFP vs 41.7% for Hsp40 ($p < 0.001$) in the striatum, and 47.5% for GFP vs 9.2% for Hsp40 ($p < 0.001$) in the cortex (Figs. 1A,C). This difference in the effectiveness of Hsp40 between the two brain areas may be due to differences in the expression levels of its partner Hsp70. Although whether polyQ inclusion bodies themselves are cytotoxic or cytoprotective has been controversial [36], we assume that suppression of inclusion body formation by QBP1 and Hsp40 can be regarded as a therapeutic effect, since they act by preventing the initial toxic misfolding of the polyQ protein and promoting its refolding, respectively [15,16,19,20]. Therefore, these results collectively demonstrate that QBP1 and Hsp40 exert therapeutic effects in polyQ disease mouse neurons, via their widespread and long-term expression using AAV5.

AAV5-Hsp40 Improves Neurological Phenotypes of polyQ Disease Mice

Since our above results demonstrated that AAV5-QBP1 and AAV5-Hsp40 inhibit accumulation of the pathogenic polyQ protein into inclusions, we next tested whether this could also lead to amelioration of the neurological phenotypes of R6/2 mice. For this purpose we injected AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40 into both sides of the striatum of P7 R6/2 mice, and analyzed their effects on various neurological phenotypes of these mice.

To evaluate motor impairments of R6/2 mice, we first tested their forced locomotor activity using the rotarod. Although the reduction in rotarod performance of R6/2 mice compared with wild-type (WT) mice becomes evident by 7 weeks of age, the performance of AAV5-QBP1 injected and AAV5-Hsp40 injected R6/2 mice did not significantly differ from AAV5-GFP injected mice (Fig. 2A). We further tested the effect of these viruses on spontaneous locomotor activity. R6/2 mice demonstrated significantly less open-field activity and rearing compared with WT mice as early as 5 weeks of age, which further decreased by 8 weeks. Although we could not detect a significant improvement in AAV5-QBP1 injected mice, AAV5-Hsp40 injected R6/2 mice exhibited significantly higher open-field activity and rearing compared with AAV5-GFP injected mice at 8 weeks of age (Activity: GFP 134.8 ± 10.2 vs Hsp40 196.4 ± 8.4 counts/min, $p < 0.001$; Rearing: GFP 2.0 ± 0.44 vs Hsp40 6.3 ± 0.67 counts/min, $p < 0.001$) (Figs. 2B,C). These results demonstrate the therapeutic effect of AAV5-Hsp40 on the decreased spontaneous locomotor activity of R6/2 mice.

We also tested the effect of each virus on the grip strength abnormality of R6/2 mice. Grip strength of R6/2 mice was significantly weaker than that of WT mice at 9 weeks of age, and further weakened by 12 weeks of age. AAV5-QBP1 injection did not have a significant effect on grip strength at either age. In contrast, AAV5-Hsp40 injected R6/2 mice exhibited significantly

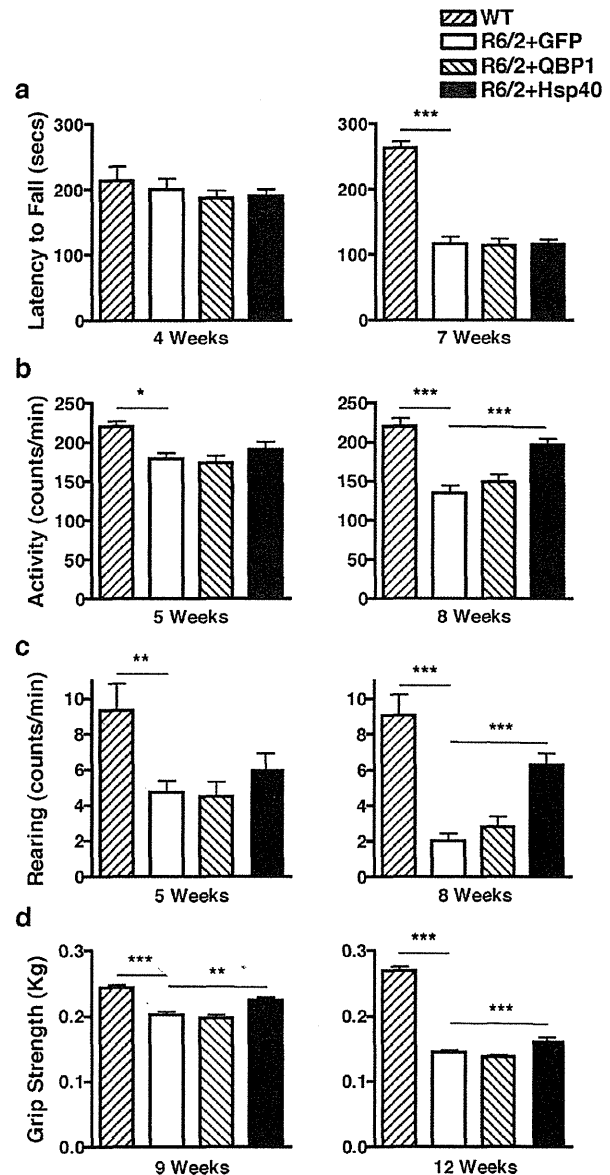


Figure 2. AAV5-Hsp40 improves spontaneous locomotor activity and grip strength abnormalities of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Rotarod performance measured at 4 (left) and 7 weeks (right) of age. (B, C) Open-field activity (B) and rearing (C) measured at 5 (left) and 8 weeks (right) of age. (D) Grip strength measured at 9 (left) and 12 weeks (right) of age. Values represent mean \pm SEM, $n \geq 9$ mice ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). doi:10.1371/journal.pone.0051069.g002

greater grip strengths than AAV5-GFP injected mice both at 9 weeks (GFP 0.202 ± 0.005 vs Hsp40 0.225 ± 0.005 Kg, $p < 0.01$) and 12 weeks of age (GFP 0.145 ± 0.003 vs Hsp40 0.165 ± 0.003 Kg, $p < 0.001$), demonstrating the therapeutic effect of AAV5-Hsp40 (Fig. 2D).

We also evaluated the body weight loss of AAV5-injected R6/2 mice. AAV5-GFP injected R6/2 mice demonstrated significantly lower body weights than WT mice after 9 weeks of age. The

weights of AAV5-QBP1 injected R6/2 mice were almost indistinguishable from AAV5-GFP injected mice at each time point. In contrast, the weights of AAV5-Hsp40 injected R6/2 mice showed a significant improvement compared to AAV5-GFP injected mice between 9 and 13 weeks of age (Fig. 3A). These results demonstrate the therapeutic effect of AAV5-Hsp40 also against the weight loss of R6/2 mice.

We then assessed the effect of AAV5 injections on the lifespan of R6/2 mice. The survival of AAV5-QBP1 injected R6/2 mice (median lifespan 100 days) was not significantly different from AAV5-GFP injected mice (median lifespan 95 days). In contrast, AAV5-Hsp40 injection resulted in a rightward shift of the survival curve to a median lifespan of 112 days, demonstrating the therapeutic effect of AAV5-Hsp40 on the decreased survival of R6/2 mice (Fig. 3B). Taken together, these results clearly demonstrate that AAV5-Hsp40 significantly improves many neurological phenotypes of R6/2 mice.

AAV5-Hsp40 Inhibits Inclusion Body Formation also in Virus Non-infected Neurons of polyQ Disease Mice

When analyzing the effect of the viruses on inclusion body formation in R6/2 mice (Fig. 1), we suspected that on the AAV5-Hsp40 injected side, not only virus infected neurons, but even virus non-infected neurons appeared to have fewer inclusion bodies than those on the AAV5-GFP injected side. To clarify our suspicion, we focused on the virus non-infected neurons that are not stained with Hsp40 or GFP antibodies (see Materials and Methods and Fig. S3), and reanalyzed inclusion body formation in the virus non-infected neurons. On the AAV5-QBP1 injected side, virus non-infected neurons showed a similar rate of inclusion body formation as non-infected neurons on the AAV5-GFP injected side, in both the striatum and cortex, as expected (Fig. 4). Surprisingly, we found that on the AAV5-Hsp40 injected side, virus non-infected neurons had strikingly fewer inclusion bodies compared with non-infected neurons on the AAV5-GFP injected side in both the striatum (GFP side 69.0% vs Hsp40 side 49.8%, $p < 0.01$) and cortex (GFP side 59.1% vs Hsp40 side 39.6%,

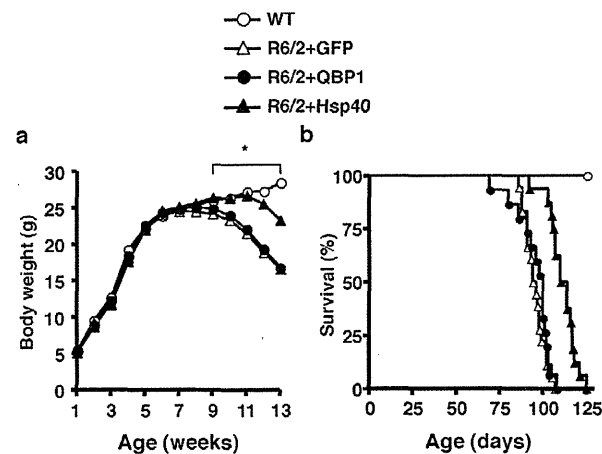


Figure 3. AAV5-Hsp40 improves body weight loss and extends the lifespan of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Body weight measured weekly. Values represent the mean ($*p < 0.05$, R6/2+GFP vs R6/2+Hsp40). (B) Survival ($p < 0.0001$, R6/2+GFP vs R6/2+Hsp40, Log-rank test). In both (A) and (B), $n \geq 9$ mice. doi:10.1371/journal.pone.0051069.g003

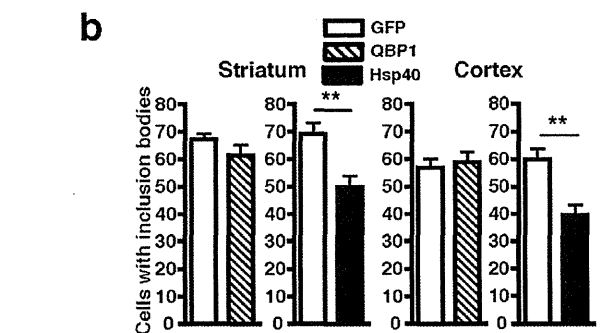
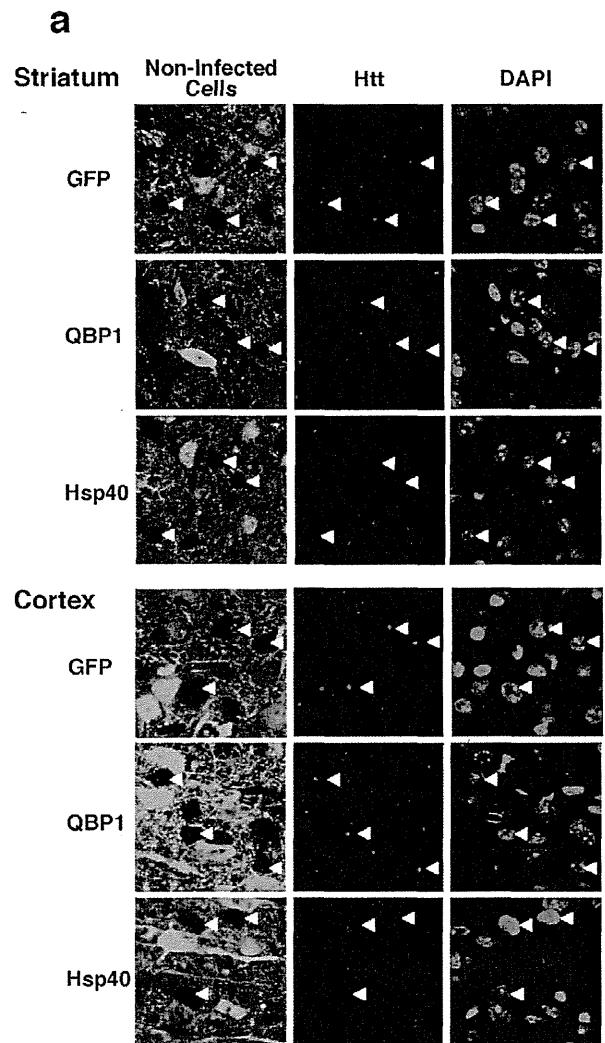


Figure 4. AAV5-Hsp40 also inhibits polyQ inclusion body formation in virus non-infected neurons of polyQ disease mouse brains. Htt inclusion body formation in 8 week old R6/2 mouse brains injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 on the other side, was analyzed as in Fig. 1, but in the virus non-infected cells on each side of the brain. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. Representative virus non-infected cells in each panel are indicated by

white arrowheads. (B) Inclusion body formation in virus non-infected cells on the AAV5-QBP1 injected side and AAV5-Hsp40 injected side in the striatum (left) and cortex (right). Data are shown as means \pm SEM of ≥ 6 fields of view, in which over 180 cells were counted (** $p < 0.01$). Representative results of two mice analyzed are shown.
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$p < 0.01$). The degree of inhibition was not as robust as in AAV5-Hsp40 infected neurons, but was still significant. These results raise a possibility that Hsp40 can exert a non-cell autonomous therapeutic effect on virus non-infected neurons in the brains of R6/2 mice, which is not observed with QBP1.

Hsp40 Inhibits Secretion of Pathogenic polyQ Proteins from Cultured Cells

We next aimed to elucidate the mechanism by which Hsp40 exerts its non-cell autonomous therapeutic effect in the brains of R6/2 mice. Recent studies suggest that prion-like cell-cell transmission of aggregation-prone proteins via their release from cells and subsequent uptake into neighboring cells, is involved in the spreading of neuropathology in the polyQ diseases as well as other neurodegenerative diseases [8–11]. We therefore hypothesized that Hsp40 may inhibit secretion of the pathogenic polyQ protein from cells to exert its non-cell autonomous therapeutic effect.

We used a cell culture model to test whether Hsp40 could inhibit secretion of a pathogenic polyQ protein from cells. An expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag (Q81-CFP-V5) was co-expressed together with the GFP control, QBP1, or Hsp40 in Neuro2A cells. Twenty-four h later, the culture media were replaced with fresh media to remove all of the dead cells, and after a further 6 h of incubation, culture media were collected and concentrated using centrifugal filters, and subjected to Western blot analysis. Q81-CFP-V5 was detected in the culture medium of cells (Fig. 5A), suggesting that pathogenic polyQ proteins are secreted from cells. In cells co-expressing QBP1, the amount of Q81-CFP-V5 detected in the culture medium was similar to that in cells co-expressing GFP. In contrast, Neuro2A cells co-expressing Hsp40 showed $\sim 40\%$ less Q81-CFP-V5 in the culture medium compared with cells co-expressing GFP, suggesting that Hsp40 inhibits secretion of the pathogenic polyQ protein from cells (Figs. 5A,B). Furthermore, siRNA-mediated knockdown of endogenous Hsp40 increased the secretion of Q81-CFP-V5 by $\sim 40\%$ compared with cells treated with a control siRNA (Figs. 5C,D), indeed confirming that Hsp40 inhibits polyQ protein secretion. These results imply that inhibition of secretion of the polyQ protein from cells by Hsp40 results in a non cell-autonomous therapeutic effect in R6/2 mouse brains, possibly via inhibition of the cell-cell transmission of the pathogenic polyQ protein.

Discussion

In this study we show for the first time that viral vector-mediated expression of a molecular chaperone, namely Hsp40 significantly improves the neurological phenotypes of a mouse model of the polyQ diseases. Although a recent study reported the effectiveness of another Hsp40 family member, HSP1a (DNAJB2a) in R6/2 mice, this study cannot be directly translated to a therapy since it was performed by the crossing of transgenic mice [30]. In addition, although lentiviral vector-mediated delivery of DNAJB2a to a polyQ disease rat model has been investigated [37], it did not demonstrate the therapeutic effect on the neurological phenotypes, perhaps because of the limited spread of lentiviruses. We

successfully overcame these above problems by using AAV, which infects a widespread area of the brain, and is already used in human patients [38].

We did not detect significant therapeutic effects of AAV5-Hsp70 on R6/2 mice unlike AAV5-Hsp40, possibly due to the very low infection rate of our AAV5-Hsp70 (data not shown), or differences in the effectiveness of Hsp40 and Hsp70 against mutant htt. Indeed, previous studies examining the effect of Hsp70 in R6/2 mice have shown no or very modest therapeutic effects [28,29]. Furthermore, a cell culture study demonstrated that Hsp40 family members are effective against the toxicity of mutant htt, while Hsp70 family members are ineffective [35]. Taken together, these studies indicate that Hsp40 family members may be more effective than Hsp70 family members against the toxic effects of mutant htt.

We surprisingly found that AAV5-Hsp40 inhibits inclusion body formation also in virus non-infected cells (Fig. 4), suggesting a non cell-autonomous therapeutic effect. Aggregation prone proteins that cause neurodegenerative diseases including pathogenic polyQ proteins, as well as α -synuclein which causes Parkinson's disease, and tau which causes the tauopathies have recently been suggested to be transmitted between cells, and this may be the mechanism leading to the progressive spread of neuropathology in these diseases [8–11]. We detected a significant amount of the pathogenic polyQ protein in the culture medium of Neuro2A cells (Fig. 5A), suggesting its cell-cell transmission, which is compatible with previous studies [39,40]. We further found that Hsp40 suppresses secretion of the pathogenic polyQ protein from cells (Fig. 5), suggesting that it may inhibit the cell-cell transmission of the pathogenic polyQ protein. The non-cell autonomous therapeutic effect of Hsp40 may also involve other mechanisms, such as (1) AAV5-Hsp40 infected neurons may create a better environment for contacting non-infected neurons [41] or (2) Hsp40 itself may be secreted to exert therapeutic effects on neighboring non-infected neurons, as is suggested for Hsp70 [42].

We were unable to detect the therapeutic effect of AAV5-QBP1 on the neurological phenotypes of R6/2 mice, although we successfully detected its inhibition of inclusion body formation. However, we and others have previously shown that expression of QBP1 exerts therapeutic effects on the neurological phenotypes of *Drosophila* and mouse models of the polyQ diseases [16,17,43]. Since the infection efficiency of all of the viruses used in this study was quite low ($\sim 30\%$), the extent of neurons expressing QBP1 in the R6/2 mouse brains was probably insufficient to exert a detectable effect on the phenotypes. On the other hand, in the case of AAV5-Hsp40, inhibition of polyQ protein secretion which should lead to an increase in the number of rescued neurons, likely contributed to its improvement of the neurological phenotypes. Other possibilities may also contribute to their varying effects, for example (1) Hsp40 is more effective than QBP1 in inhibiting polyQ protein misfolding/aggregation, and (2) Hsp40 can also support the degradation of misfolded proteins [19], while QBP1 cannot.

In this study we demonstrate a therapeutic strategy against the polyQ diseases, using AAV5-Hsp40, which has great potential for clinical application, since AAVs are safe and are widely utilized in clinical trials [38]. We further suggest a novel therapeutic mode of action of Hsp40, namely suppression of pathogenic polyQ protein secretion from cells, which may consequently suppress its cell-cell transmission. Since the transmission of aggregation-prone proteins is thought to be involved also in other neurodegenerative diseases, Hsp40 may exert a non-cell autonomous therapeutic effect on these other diseases. Elucidation of how Hsp40 inhibits polyQ protein secretion should reveal new therapeutic targets and

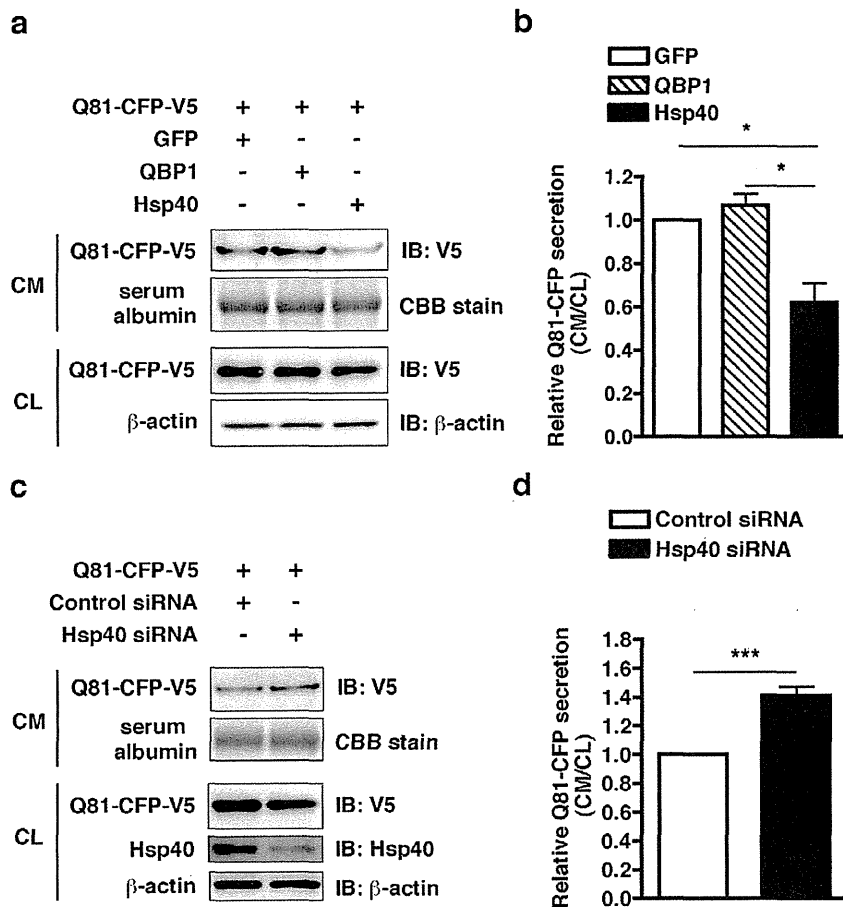


Figure 5. Hsp40 inhibits polyQ protein secretion in cultured cells. (A) Neuro2A cells were co-transfected with plasmids expressing Q81-CFP-V5 and either GFP, QBP1, or Hsp40, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein. (B) Relative amounts of Q81-CFP-V5 secreted into the culture media, calculated from the band intensities in (A), with the amount of Q81-CFP-V5 secreted from cells co-expressing GFP set to 1. (C) Neuro2A cells were transfected with a plasmid expressing Q81-CFP-V5 and siRNA against Hsp40 or a control siRNA, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein, and with an Hsp40 antibody. The loading controls are as in (A). (D) Relative amounts of Q81-CFP-V5 secreted into the culture media, calculated from the band intensities in (C). In (A) and (C), serum albumin is shown as a loading control for the culture media, and β -actin as a loading control for the cell lysates. In (B) and (D), data are shown as means \pm SEM of \geq four independent experiments ($*p < 0.05$, $***p < 0.001$).

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strategies for neurodegenerative diseases caused by aggregation-prone proteins.

Materials and Methods

Viral Vectors

Adeno-associated virus type 5 (AAV5) vector plasmids contained an expression cassette with a human cytomegalovirus enhancer/chicken β actin (CAG) promoter followed by the first intron of human growth hormone, target cDNA (either a tandem repeat of QBP1 fused to GFP [14], human Hsp40 (DNAJB1) [44], or GFP), and a simian virus 40 polyadenylation signal sequence, all positioned between the inverted terminal repeats of the AAV5 genome. AAV5 vectors were produced using the AAV5 plasmid, the AAV5 helper plasmid containing the rep and cap sequences from AAV5, as well as the pHelper plasmid from the AAV Helper-Free System containing the E2A, E4, and VA RNA genes of the adenovirus genome (Stratagene, La Jolla, CA). HEK293 cells were

co-transfected with the AAV5 plasmid and two helper plasmids by the calcium phosphate method. Seventy-two h later, the cells were harvested and subjected to three rounds of freeze-thaw lysis. AAV5 vectors were then purified by two rounds of cesium chloride density gradient centrifugation. Vector titers were estimated by quantitative DNA dot-blot hybridization to be ~ 0.2 – 1.6×10^{13} genome copies/ml.

Animals

All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, and performed in accordance with the guidelines. Mice transgenic for human *huntingtin* exon 1 with approximately 150 CAG repeats (strain R6/2) [33] were obtained from the Jackson Laboratory (Bar Harbor, ME), and maintained on a B6CBAF1 background. Genotypes were analyzed and CAG repeat numbers of the transgenic mice were confirmed to be

similar by PCR as previously described [33]. Mice were housed on a 12-hour light/dark cycle, with food and water provided *ad libitum*. At least nine male R6/2 mice per group and wild-type littermate (WT) controls were used for the phenotype analyses, and two R6/2 mice were used for the inclusion body analyses.

AAV Injections

P7 old R6/2 mice were stereotaxically injected with 1 μ l of virus solution (AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40) into the striatum (coordinates 1 mm anterior to bregma, 2.25 mm lateral to the midline, and 3 mm ventral to the skull surface) at a rate of 0.1 μ l/min using a 10 μ l Hamilton syringe (Hamilton Company, Reno, NV) and an infusion pump (KD Scientific, Holliston, MA). For inclusion body analyses, AAV5-GFP was injected into one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 into the other side. For phenotype analyses, the same virus was injected into both sides of the striatum.

Mouse Phenotype Analyses

For rotarod analysis, mice were tested at 4 and 7 weeks of age on an accelerating rotarod apparatus (Ugo Basile, Comerio, Italy) set to accelerate from 4 to 40 rpm over a period of 300 seconds. The time it took for each mouse to either fall off the rod or cling onto the rod for one full rotation was recorded. Mice were tested on the rod for three consecutive days, with three trials per day. The first day was regarded as training, and only the data from the second and third day were used. The highest and lowest values were excluded, and the middle four values were averaged. Grip strength analysis was performed at 9 and 12 weeks of age using a grip strength meter (Muromachi Kikai, Tokyo, Japan). Mice were placed gently by their tail on the metal grid of the meter so that they grip the grid with both forelimbs and hindlimbs, at which point they were pulled back gently with their tails, exerting a tension that is measured by the meter. Five trials were performed for each mouse, and the highest and lowest values were excluded and the middle three values were averaged. To measure open-field activity and rearing, mice at 5 and 8 weeks of age were tested using a spontaneous locomotor activity monitor (Supermex, Muromachi Kikai, Tokyo, Japan), consisting of an acrylic box of dimensions 40 cm \times 28 cm \times 31 cm with an activity sensor placed at the top and rearing sensors placed at the sides of the box. Mice were left in the box for a total of 15 min, and their activity count and rearing frequency were measured. For body weight analysis mice were weighed weekly. Mice were followed until their deaths in order to calculate their lifespans, a widely accepted and valuable parameter to assess therapeutic effects in these mice, which was approved by the institution's Animal Ethics Committee.

Tissue Preparation and Immunohistochemical Analyses

Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg), and then perfused intercardially with saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed, post-fixed in 4% PFA in PBS at 4°C overnight, and then cryoprotected in 30% sucrose in PBS at 4°C for 24 h. Frozen 10 μ m sections were cut using a cryostat. For immunohistochemical analysis, sections were blocked in PBS containing 5% goat serum and 0.1% Triton X-100 for 1 h at room temperature. The sections were then incubated with a mouse anti-huntingtin antibody (1:250; MAB5374, Millipore, Billerica, MA), and either a rabbit anti-Hsp40 antibody (1:500; SPA-400, Enzo Life Sciences, Farmingdale, NY) or a rabbit anti-GFP antibody (1:250; A11122, Invitrogen, Carlsbad, CA) at 4°C overnight, followed by an Alexa Fluor 596-conjugated goat anti-mouse IgG antibody and an Alexa Fluor 488 goat anti-rabbit IgG antibody

(1:1000, Invitrogen) for 1 h at room temperature. Sections were mounted with Slowfade Gold antifade reagent with DAPI (Invitrogen) and examined using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). The average fluorescence intensity of representative cells that were regarded as either "infected" or "non-infected" in each sample were measured using the Image J software, confirming that the two population of cells can readily be distinguished in the images (Fig. S3).

Cell Culture, Transfection and Western Blot Analysis

Neuro2A cells (obtained from ATCC) were grown and maintained in DMEM supplemented with 10% (v/v) FBS. Cells were plated on a 35-mm dish at a density of 4×10^5 cells per dish on the day before transfection. For the overexpression experiment, a plasmid vector encoding a tandem repeat of QBP1 fused to GFP, human Hsp40 [44], or GFP was transiently co-transfected with the Q81-CFP-V5 vector encoding an expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag, using Lipofectamine LTX with PLUS reagent (Invitrogen). For the knockdown experiment, siRNA targeted against mouse Hsp40 (Santa Cruz Biotechnology, Santa Cruz, CA) or a control siRNA (RNAi Inc, Tokyo, Japan) was cotransfected with the Q81-CFP-V5 vector using Lipofectamine 2000 reagent (Invitrogen). At 24 h after transfection, culture media were replaced with fresh media, and after a further 6 h of incubation, culture media were collected and whole cell lysates were prepared with 1% Triton X in tris-buffered saline. For Western blot analysis, the culture media were concentrated using 30 kDa cutoff Amicon Ultra filters (Millipore). Q81-CFP-V5 in the concentrated culture media and whole cell lysates was separated using 10% SDS-PAGE gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were incubated overnight with an HRP-conjugated anti-V5 antibody (1:2500, Invitrogen) at 4°C. The HRP signal was visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Rockford, IL), captured with a LAS-3000 mini CCD imaging system (Fujifilm, Tokyo, Japan), and band intensities were quantified using the Image J software. For the siRNA experiments, the membranes were then stripped and incubated with a rabbit anti-Hsp40 antibody (1:5000, SPA-400, Enzo Life Sciences Inc, Farmingdale, NY) at 4°C followed by an HRP-conjugated anti-rabbit IgG secondary antibody (1:10000, Thermo Fisher Scientific), and visualized as above.

Statistical Analyses

For the rotarod, open-field activity, rearing, grip strength and weight data, statistical analyses were performed by using one-way analysis of variance followed by Tukey's multiple comparison test to assess for significant differences between individual groups. The survival data was analyzed using the Log-rank test. For the inclusion body formation and Western blot analyses, Student's *t*-test was used. For all analyses, $p < 0.05$ was considered as significant.

Supporting Information

Figure S1 AAV5 injection into the mouse striatum at P7 results in widespread expression of the transgene. R6/2 mice at P7 were injected in the right striatum with 1 μ l of AAV5-QBP1, and 2 weeks later the expression of QBP1 was analyzed by immunohistochemistry. This widespread expression of QBP1 throughout the brain lasts for at least 13 weeks (data not shown). (PDF)

Figure S2 AAV5-QBP1 inhibits polyQ inclusion body formation in virus infected neurons of polyQ disease