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Gene expression profile in fibroblasts of Huntington's disease patients and controls

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ABSTRACT

Huntington's disease is an inherited disorder caused by expanded stretch of consecutive trinucleotides (cytosine–adenosine–guanine, CAG) within the first exon of the huntingtin (*HTT*) gene on chromosome 4 (p16.3). The mutated huntingtin (mHTT) gains toxic function, probably through mechanisms that involve aberrant interactions in several pathways, causing cytotoxicity. Pathophysiology of disease involves several tissues; indeed it has been shown that there is a broad toxic effect of mHTT in the peripheral tissue of patients with HD, not only in the central nervous system. In this study we compared gene expression profiles (GEP) of HD fibroblasts and matched controls using microarray technology. We used RT-PCR to test the consistency of the microarray data and we found four genes up-regulated in HD patients with respect to control individuals. The genes appear to be involved in different pathways that have been shown to be perturbed even in HD models and patients. Although our study is preliminary and has to be extended to a larger cohort of HD patients and controls, nevertheless it shows that gene expression profiles seem to be altered in the fibroblasts of HD patients. Validation of the differential expressions at the protein level is required to ascertain if this cell type can be considered a suitable model for the identification of HD biomarkers.

1. Introduction

Huntington's disease (HD, OMIM #143100) is a rare and late onset hereditary neurodegenerative disorder characterized by progressive symptoms and prevalence, in the Caucasian population, of about 1/10,000.

The disease is clinically characterized by motor symptoms, cognitive impairment and psychiatric disturbances, which generally appear between 30 and 50 years, but a broad variability in the age of onset has been also described. The course of the pathology is progressive with life expectancy of about 15–20 years after the onset.

In juvenile HD (JHD) symptoms appear before the age of 20 years and the course is more rapidly progressive; in a recent study it has been shown that the mean proportion of JDH cases is less than 5%, but frequency varies in different populations [1].

HD is inherited in autosomal dominant manner and it presents full penetrance, with the offspring of an individual with a mutant allele having a 50% chance of inheriting the disease-causing allele.

The clinical characteristics of the disease are known since 1872, but the gene was mapped only in 1983, on 4p16.3 [2] and was isolated in 1993 [3]. *HTT* gene encodes for a 350kDA ubiquitously expressed protein called huntingtin (HTT). The causative mutation is an anomalous expansion of a tract of CAG trinucleotide repeats within the coding sequence of the gene, leading to an abnormally expanded polyglutamine tract in huntingtin. There is a strong inverse relationship between the age of onset of HD and the number of CAG repeats: longer repeats are correlated with an earlier age of onset [4,5]. Normal individuals have less than 36 repeats, commonly 15–25. Alleles of 40 CAG and above are fully penetrant and cause Huntington's disease, while individuals with 27-35 repeats do not have the disease but those alleles are potentially unstable during reproduction [6,7]. Indeed, alleles of 36–39 (CAG) are incompletely penetrant and can be found in affected individuals as well as in individuals who do not show clinical symptoms so those alleles confer an increasing risk of developing Huntington disease [8].

Like other diseases caused by CAG repeats, HD exhibits anticipation, an earlier disease onset from generation to generation [9]. The repeat instability is more likely observed during spermatogenesis than ovogenesis [10].

As for many neurodegenerative disorders, conventional therapeutics function as symptom relief, and have no effects on disease progression.

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Many efforts have been made to:

1) Improve the knowledge of the pathogenic pathway, to target related molecules.
2) Identify peripheral markers that can be useful for biological monitoring of the disorder and to test the effectiveness of emerging therapies.

The biological function of the HTT protein is not fully understood and it would be useful to dispose of *in vitro* tools for the evaluation in depth of its role in neuronal cells since the genetic mutation in *HTT* gene defines the pathology, but differential gene expression could be useful to indicate the activation of abnormal processes.

In neurons affected by neurodegenerative disease significant variations in morphology, physiology and function have been observed that are related to discrepancies in gene expression profiles of affected cells [11]. Moreover it has been shown that there is a broad toxic effect of mHTT in the peripheral tissues of patients with HD [12]. Hence, the study of gene expression profiles could provide information about the onset and the progression of the disease.

Microarray technology is a valuable tool for quantifying the trascriptome in a unique experiment and it allows to identify differences in gene expression by comparing pathological cells and normal ones. Several gene expression profiling (GEP) studies have been performed in post-mortem HD brain, lymphocytes from HD patients and on transgenic mouse models and cellular models of HD (reviewed by Cha [13]).

The latest studies of gene expression in HD have been performed by Dalrympe et al. [14], Anderson et al. [15], Chang et al. [16], and Krzyszton-Russian et al. [17] in blood cells from HD patients, whereas Pouladi et al. [18] identified a reduced IGF-1 expression in skin-derived fibroblasts from HD patients. Furthermore, del Hoyo et al. [19] showed a decreased activity of catalase in skin fibroblast cultures from HD patients, Mazzola and Sirover [20] reported a decrease of the GAPDH glycolytic activity in fibroblasts from HD patients and Seo et al. [21] showed the altered activity of the ubiquitin proteasome system (UPS) in the same cell type.

We choose to analyze fibroblast's gene expression because mHTT is expressed ubiquitously, therefore molecular changes detected in fibroblasts may reflect peripheral processes promoted by mHTT. The study of cultured fibroblasts has some drawbacks: gene expression profiles are influenced by culture conditions, age of cultures and growth phase. However fibroblasts can be easily obtained and cultured, allowing the extraction of high quality RNA in a simple and reliable way; this is not necessary true for post-mortem brain tissue because RNA has a fragile nature and it is degradable. Last but not least, fibroblasts have the same embryonic origin as neurons.

In this study we compared the gene expression profile of HD fibroblasts and healthy ones to identify differentially expressed genes, which may be used as biomarkers for the disease.

To the best of our knowledge, there is not any published study that had solely investigated the gene expression profile in dermal fibroblasts from HD patients.

2. Materials and methods

2.1. Cell lines

Dermal fibroblast cell lines from six HD individuals and five normal controls were collected for this study.

HD fibroblasts have been collected from punch biopsies of patients of the Neurology Unit of University of Brescia, Italy. Punch biopsies have been obtained even from healthy volunteers of the same unit.

Subjects with inflammatory or infective conditions were excluded.

All patients and volunteers gave their written informed consent.

Besides, we used GM04476 and GM04799 fibroblast HD cell lines obtained from the Coriell Cell Repositories (CRC), Camden, NJ, and two additional cell lines as controls deriving from CRC, GM04735 and GM04781. The skin biopsy samples were placed in phosphate buffer saline (PBS) solution prior to being cut into small pieces and transferred into a second dish containing RPMI with 20% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 U/mL penicillin and incubated at 37 °C in a humidified chamber with 5% CO_2. All fibroblast cells were cultured in Eagle's minimum essential (MEM) (Euroclone Life Sciences) supplemented with 10% FBS (HyClone), 100 mg/mL streptomycin, and 100 U/mL penicillin and incubated at 37 °C in a humidified chamber with 5% CO_2. Cells were harvested once they reached confluence by treating with trypsin (0.05% trypsin with 0.25% EDTA, Invitrogen). RNA samples were extracted when all cell cultures were at passages 8–10.

2.2. RNA isolation

Total RNA was isolated from cells using TRIzol (Invitrogen, Life Technologies), followed by RNeasy Minin Kit (QIAgen, Venlo, The Netherlands) and eluted in 30 μL RNase free water, according to the manufacturer's protocol. The RNA was quantified using the NanoDrop ND-1000 spectrophotomer (Thermo Scientific, Wilmington, DE) and the quality of the total RNA was determined electrophoretically by the RNA Nano Assay Chip (RNA 6000 Nano Kit) on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). We normally obtain a total RNA with a 260/280-absorbance ratio of two roughly.

2.3. Microarray hybridization

RNA was prepared according to manufacturer's one cycle target labeling procedure (Affymetrix, Inc, Santa Clara, CA), according to the standard protocol described in the Affymetrix GeneChip Expression Analysis Manual.

Quickly, cDNA was generated using GeneChip® Expression-3′ Amplification One-Cycle cDNA Synthesis Kit with a starting amount of 2 μg of high-quality total RNA. The labeled cRNA obtained after GeneChip® Expression 3′-IVT Labeling Kit was cleaned, quantified and after fragmentation 15 μg of cRNA was hybridized on Affimetrix GeneChip® Human Genome U133 Plus 2.0 (HG-U 133 plus 2.0). This array is a single GeneChip composed of more than 54,000 probe sets representing 39,000 well-substantiated human gene transcripts.

The GeneChip was scanned and data extracted using GeneChip scanner 3000 7G (Affimetrix, Santa Clara, CA).

2.4. Data processing and statistical analysis

Raw data (CEL files) were background corrected, normalized and summarized into probe set expression values using the robust multichip average (RMA) algorithm within Bioconductor v2.7 using OneChannelGUI package on R 2.11.0 environment [22].

Normalized data were filtered by applying an interquartile (IQR) filter (IQR < 25% of mean total IQR) to remove the non-significant probe sets (i.e., not expressed and those not changing) [23]. To assess differential expression, an empirical Bayes method [24] was used to moderate the standard error of the estimated log-fold changes together with a false discovery rate (FDR) correction of the *P*-value [25].

The list of differentially expressed genes was selected considering a $P < 0.01$ together with an absolute fold-change threshold of 2.

All microarray data are MIAME compliant and the raw data have been deposited in the MIAME compliant GEO database (accession number GSE45516).

Subsequently, the dataset of differentially expressed genes was submitted to the GeneOntology (GO) database, via OntoExpress software [26] in order to identify the most representative categories, such as cellular compartment and biological processes within a more meaningful biological frame.

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2.5. Gene validation by real-time PCR

Real-time PCR was performed according to the recommendations supplied by Applied Biosystems.

RNA was reverse transcribed (RT) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's protocol. The starting amount was 20 ng of cDNA sample, 12.5 μL of TaqMan PCR Master Mix (Applied Biosystem), 1.25 μL of TaqMan Universal TaqMan Probe in a total volume of 25 μL. The ABI PRISM 7500 Detection System was used to detect amplification levels and was programmed for an initial step at 95C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Real-time PCR analysis was performed on individual samples (i.e., without pooling) in triplicate. Oligonucleotides for real time PCR were purchased from Applied Biosystems as 'AssaysOnDemand'® to detect expression of *APC*, *CTNNB1*, *ROCK1*, *ROCK2*, *SSH1*, *PLCB4*, *UBE2D3*, *DN1LM* and *CDC42EP2* genes. The abundance of the target mRNAs was calculated relative to a reference mRNA (ATP5B).

Relative expression ratios were calculated as $R = 2^{(\mathrm{Ct(ATP5B)} - \mathrm{Ct(Test)})}$, where Ct is the cycle number at the threshold and the test stands for the tested mRNA. The confidence interval was fixed at 95%.

3. Results

3.1. Microarray analysis of HD fibroblasts vs. normal controls

The microarray analysis was carried out on dermal fibroblast cell lines derived from six HD patients and three normal individuals, age- and gender-matched. Moreover, we tested the gene expression profile of specific selected differentially expressed genes using real-time PCR in four cell lines of CRC derivation, two of them from HD patients, and two used as further controls, and in five normal individuals (Table 1). The number of repeats was established by PCR analysis of the region encompassing the CAG repeat, followed by fragment sizing through capillary electrophoresis [27]. PCR products were analyzed using the ABI Prism 3130 DNA analyzer instrument and GeneScan (Applied Biosystem). The variation of repeat size matches to the amplitude of Genescan profile and was determined by calculating the number of peaks above 10% of the maximum fluorescent peak intensity.

As shown in Table 1, we found individual repeat length differences between blood cells and fibroblasts. A great number of Authors discussed the degree of somatic repeat instability in HD as in other pathologies associated with inherited unstable DNA triplets (e.g. myotonic dystrophy 1, DM1). It has been shown that higher levels of somatic mosaicism can be found in disease related tissues, like muscle in DM1 [28] and brain in HD [29]. The molecular mechanisms underlying this dynamic mutation are still poorly understood.

Oligoarray hybridization, washes and scanning were carried out as described in the Materials and methods section. GenMapp software was used to identify the pathways where genes up and down-regulated play a role.

The microarray data were fitted out according to minimum information about a microarray experiment (MIAME) recommendations and deposited in the public GEO database. Hereafter, the terms "up-regulated" will be used to denote genes that are expressed more than two-fold ($P < 0.01$) in HD fibroblasts than in the control cells. On the contrary, genes expressed more than two-fold in control cells are defined "down-regulated". Our study identified 261 up-regulated and 9 down-regulated genes.

The results of Onto-Express analysis are shown in Tables 2 and 3. Table 2 illustrates the Gene-Ontology cellular districts for both up- and down-regulated genes, whereas Table 3 shows the biological processes for both up- and down-regulated genes.

3.2. Validation of microarray data

To identify a series of genes that could give new insights into pathological mechanism of HD and identify potential biological markers, candidate genes differentially expressed in HD patients and controls were tested by real-time PCR to test the consistency of the microarray data.

The real-time PCR data obtained from triplicate experiments for each cell line were quantified and the expression ratios of genes between HD patients and controls were calculated and compared with the array data. This analysis has been carried out on nine genes: *APC*, *CDC42EP2*, *CTNNB1*, *DNM1L*, *PLCB4*, *ROCK1*, *ROCK2*, *SSH1*, and *UBE2D3*. Some of these genes have been chosen mainly based on the high level of differential up-regulation (*PLCB4*, *APC*) or down-regulation (*SSH1*, *CDC42EP2*); others are due to their possible involvement in HD pathogenesis, as indicated by previous studies (*UBE2D3*, [30]; *ROCK1*, *ROCK2*, [31]; *DNM1L*, [32]; *CTNNB1*, [33]) (Table 4).

Differences in gene expression levels were evaluated using a relative quantitative method as described by Pfaffl [34] and calculated using the $2^{-\Delta\Delta CT}$ method. All expression values were normalized with that of the endogenous control housekeeping transcript ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*).

All expression values were statistically significant ($P < 0.05$, by a Student's *t*-test) and reported as mean ± standard error of the mean.

Four genes (*APC*, *PLCB4*, *ROCK1*, and *UBE2D3*) were in good agreement with the results from microarray data and are all up-regulated in HD patients with respect to control individuals (Table 4).

4. Discussion

In this study, we performed gene expression profiling (GEP) analysis on HD peripheral tissue skin fibroblasts, to assess if they could be used as an alternative cell type for biomarker studies.

GEP analysis in neurodegenerative disease is often impaired by the lack of suitable biological material from affected individuals as source of RNA. As for the majority of genes implicated in familial neurodegenerative diseases, the *HTT* gene is ubiquitously expressed and in HD evidence of systemic involvement is reported [35,36].

Table 1
Genetic characteristics of the HD patients and the controls.

Cases	Age at biopsy (years)	$(CAG)_n$ fibroblasts	$(CAG)_n$ lymphocytes	Controls	Age at biopsy (years)	$(CAG)_n$ fibroblasts	$(CAG)_n$ lymphocytes
HD1F	38	15–43	17–45	HD1C	28	Normal range	Normal range
HD2F	38	15–49	17–51	HD2C	48	Normal range	Normal range
HD3F	63	15–40	15–40	HD3C	40	Normal range	Normal range
HD4F	57	20–40	20–40	HD4C	63	Normal range	Normal range
HD5F	76	18–39	18–39	HD5C	57	Normal range	Normal range
HD6F	37	15–43	14–42	GM04735	64	17–32	NA
GM04476	57	14–53	NA	GM04781	71	13–19	NA
GM04799	47	13–42	NA				

Patients and controls are both males. CAG repeat lengths are not available (NA) for samples purchased from the Coriell Cells Repository.

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Table 2
Cellular districts and respective number of genes.

Cellular Component	Genes (*N*)	*P*
Intracellular	224	3.49E−10
Cytoplasm	138	4.65E−10
Nucleus	132	2.85E−10
Integral to membrane	29	8.70E−10
Cytoskeleton	26	1.41E−02
Golgi apparatus	25	1.17E−03
Nucleolus	16	3.22E−02
Golgi membrane	14	4.95E−03
Extracellular region	12	4.02E−02
Heterogeneous nuclear ribonucleoprotein complex	3	3.55E−02
Hemidesmosome	2	6.23E−03

Only significant cellular categories are shown ($P < 0.05$).

We used DNA microarray technology to compare gene expression profiles of fibroblasts from patients affected by Huntington disease and healthy controls and we found several genes that are differentially expressed. Validation experiments carried out by real-time PCR on a subset of nine genes confirmed that four of them, *PLCB4*, *UBE2D3A*, *APC*, and *ROCK1*, are up-regulated in HD patients.

PLCB4 gene is located in 20p12 and encodes for the phosphoinositide-specific phospholipase C β4 (PLC-β4). Thirteen distinct mammalian PLC isoforms have been identified and grouped into six families: PLC-β (1–4), PLC-γ (1–2), PLC-δ (1,3,4), PLC-ε, PLC-ζ, and PLC-η (1–2) (reviewed in Oude Weernink et al.) [37] and a recent study showed that PLC-β4 is also expressed in human skin fibroblasts [38]. The PLCβ isoforms (PLCβ1–4) are all strongly activated by $G\alpha_q$ so activation of $G\alpha_q$ results in activation of PLC-β [39]. The 1,4,5 inositol triphosphate (IP_3) is an important second messenger and its receptor (IP_3R) is an intracellular calcium (Ca^{2+}) release channel that plays an important role in neuronal Ca^{2+} signaling. A study showed that mutant HTT specifically binds to the C-terminal region of IP_3R1 and the binding causes sensitization in IP_3R1 to activation by IP_3 in medium spiny striatal neurons [40]. A study on *Htt*-null mouse embryo fibroblasts [41] showed that HTT plays a direct role in Ca^{2+} signaling by modulating IP_3R sensitivity to IP3. Moreover, it has been shown that mHTT, but not the wtHTT, enhances IP_3R1 activity in lipid bilayers [42]. The strong up-regulation of *PLCB4* gene in HD fibroblasts with respect to controls (see Table 4) could be involved in altered Ca^{2+} homeostasis and subsequently in the abnormal ultrastructural morphology of endoplasmic reticulum and mitochondria found in HD fibroblasts with respect to controls [43].

The *UBE2D3* gene (also known as *UBCH5C*) encodes for a member of the E2 ubiquitin-conjugating enzyme family. Covalent attachment of Ubiquitine (Ub) to a substrate proceeds through a multi-enzyme process consisting of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3) [44]. In the human genome there are two Ub E1s, about 35 E2s, and many hundreds of E3s [45]. The process leads to formation of a tagged poly-Ub chain, which is recognized and finally degraded by the ubiquitin–proteasome system (UPS) [46,47]. It has been shown that UBE2D3 involved is the last step in the activation of the transcription factor NF-κB, by the degradation of the inhibitor Iκ-Bα [48]. Interestingly, it has been shown that activation of the IkB kinase complex and NF-κB contributes to mHTT neurotoxicity in striatal cells from HD transgenic mice [49].

Table 3
Biological processes and respective number of genes.

Biological Process	Genes (*N*)	*P*
Transcription	59	2.49E−03
Regulation of transcription, DNA-dependent	54	6.17E−03
Cell cycle	26	6.17E−03
Response to DNA damage stimulus	19	6.42E−05
DNA repair	16	1.15E−03
Ubiquitin-dependent protein catabolic process	11	1. 68E−02
DNA recombination	10	3.37E−03

Only significant biological processes are shown ($P < 0.05$).

Table 4
Differentially expressed genes in patients and controls we selected for validation of microarray data.

Gene ID	Gene name	Protein name	Regulation in HD patients	Fold change (HD vs. controls)	Reference
5332	*PLCB4*	Phospholipase C, beta 4	Up	11.68	–
7323	*UBE2D3*	Ubiquitin protein ligase E3	Up	4.72	Branco et al., 2008 [30]
324	*APC*	Adenomatous polyposis coli protein	Up	3.53	–
6093	*ROCK1*	Rho-associated, coiled-coil containing protein kinase 1	Up	3.29	Bauer et al., 2009 [31]
19878	*ROCK2*	Rho-associated, coiled-coil containing protein kinase 2	Up	3.60	Bauer et al., 2009 [31]
10059	*DNM1L*	Dynamin 1 like protein	Up	2.40	Oliveira et al., 2010 [32]
1499	*CTNNB1*	Catenin (cadherin-associated protein), beta 1	Up	2.51	Kaltenbach et al., 2007 [33]
54434	*SSH1*	Slingshot protein phosphatase 1	Down	0.40	–
10435	*CDC42EP2*	CDC42 effector protein 2	Down	0.48	–

The former four genes listed are those who showed good agreement with the results from microarray data.

Rho-associated coiled-coil kinases (Rho-kinases or ROCKs) are serine/threonine kinases that are activated by RhoA GTPases, and are key modulators of processes involving cytoskeletal rearrangement such focal adhesion formation, cell motility and tumor cell invasion. ROCKs phosphorylate a number of proteins involved in actin filament assembly and contraction [50]. In mammals, there are two isoforms of ROCK, ROCK1 and ROCK2, which share a high degree of structural identity in their kinase domains (92%). The two Rho-kinase isoforms have different patterns of tissue expression; ROCK1 has a ubiquitous tissue distribution whereas ROCK2 expression is highest in the brain and in muscle. It has been shown that blocking the RhoA/ROCK pathway inhibits polyQ protein aggregation and decreases its toxicity in cellular models of HD [51]. A recent study showed that vimentin — a cytoskeleton protein — is specifically phosporylated by ROCK1 at Ser71 and Ser38 amino residues and that vimentin phosphorylation by ROCK1 increases polyQ aggregation [52].

Another gene we found up-regulated in HD fibroblasts is the adenomatous polyposis coli (*APC*), which encodes for a multifunctional protein involved in different processes including cell migration and adhesion, transcriptional activation, and apoptosis [53]. APC is involved in the canonical Wnt signaling pathway, also named Wnt/β-catenin pathway. In particular, APC specifically ensures that β-catenin is targeted for ubiquitination and proteosomal degradation [54]. APC has also been shown to promote β-catenin nuclear export and to act as a chromatin-associated suppressor for β-catenin target genes, thus functioning in the nucleus [55]. Involvement of Wnt signaling in neurodegeneration in general, and specifically in HD, was indicated by different lines of investigations (lately reviewed by Kalathur et al., 2012) [56]. An accumulation of β-catenin in cellular, murine and *Drosophila* models of HD and in post-mortem brain samples from HD patients has been observed, and this accumulation of β-catenin appears to be toxic, whereas its enhanced degradation reduces polyglutamine-increased neuronal toxicity [57].

In summary, further studies are required to confirm our GEP data at protein level to establish if the transcript levels of genes we found up-

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regulated deserve broad evaluation as biomarkers for HD. Moreover, our investigation needs to be extended to a larger cohort of patients and controls. Nevertheless, our study showed that skin-derived fibroblasts from HD patients seem to have altered gene expression profile with respect to healthy subjects and they might be useful to integrate knowledge about Huntington's disease.

Conflict of Interest

The Authors state that there is no conflict of interest.

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