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ORIGINAL INVESTIGATIONS

Genetic analysis of candidate genes modifying the age-at-onset in Huntington's disease

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Abstract The expansion of a polymorphic CAG repeat in the *HD* gene encoding huntingtin has been identified as the major cause of Huntington's disease (HD) and determines 42–73% of the variance in the age-at-onset of the disease. Polymorphisms in huntingtin interacting or associated genes are thought to modify the course of the disease. To identify genetic modifiers influencing the age at disease onset, we searched for polymorphic markers in the GRIK2, TBP, BDNF, HIP1 and ZDHHC17 genes and analysed

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seven of them by association studies in 980 independent European HD patients. Screening for unknown sequence variations we found besides several silent variations three polymorphisms in the *ZDHHC17* gene. These and polymorphisms in the *GRIK2*, *TBP* and *BDNF* genes were analysed with respect to their association with the HD age-at-onset. Although some of the factors have been defined as genetic modifier factors in previous studies, none of the genes encoding GRIK2, TBP, BDNF and ZDHHC17 could be identified as a genetic modifier for HD.

Keywords Huntington's disease · Age-at-onset · Genetic modifiers · Association study

Introduction

Huntington's disease (HD) is an autosomal dominant disorder that leads to a progressive loss of neurons preferentially in the striatum and cortex. The symptoms, which usually appear between 40 and 50 years of age, are cognitive defects, psychiatric disorders and motor dysfunction (Haigh et al. 2004). An expansion of a polymorphic CAG repeat in the coding region of the *HD* gene encoding huntingtin has been identified as the genetic cause of HD (The Huntington's Disease Collaborative Research Group 1993). More than 38 repeats can cause the disease with the age-at-onset being inversely correlated with the repeat length. Several studies revealed that the CAG repeat number accounts for 42–73% of the variance in the age-at-onset (Andrew et al. 1993; Brinkman et al. 1997; Stine et al. 1993). The remainder of the variance is determined by other environmental and genetic factors. In the homogeneous Venezuelan population, the additive genetic heritability of the residual age-at-onset is 38% (The U.S.–Venezuela Collaborative Research Project and Wexler 2004). A genome scan for modifiers of age-at-onset at a 10 cM density revealed suggestive evidence for linkage at 4p16, 6p21–23 and 6p24–26, respectively (Li et al. 2003). However, despite the large number of 695 individuals included in this study, there was only a power of 60% to detect LOD scores of 2.3 if the locus explains 35% of the variance in the age-at-onset. Rosenblatt et al. (2002) suggested that in addition to the repeat length a further 11–19% of the variance may be accounted for by genetic factors. Thus, several loci might be undetectable by conventional genome scans.

The genetic analysis of candidate genes, however, is an appropriate alternative to identify modifier genes. Possible candidates are, amongst others, genes encoding products interacting with wild type or mutant huntingtin. Polymorphisms in these genes, exerting no effects in unaffected individuals, could modify the course of disease. Several studies have shown an effect of a TAA repeat in the 3′ untranslated region of the glutamate receptor *GRIK2* gene (*GluR6* gene), which might explain 2–4% of the variance in the age-at-onset (Chattopadhyay et al. 2003; MacDonald et al. 1999; Rubinsztein et al. 1997). Other studies suggested a contribution of the S18Y polymorphism in the ubiquitin carboxy-terminal hydrolase L1 (*UCHL1*) gene (Metzger et al. 2006; Nazé et al. 2002), the apolipoprotein E ε2ε3 genotype (Kehoe et al. 1999), the polymorphic $(Gln\text{-}Ala)_{38}$ repeat in the transcriptional coactivator *CA150* gene (Chattopadhyay et al. 2003; Holbert et al. 2001) and gene variations in subunits of the NMDA receptors on the age-at-onset in HD (Arning et al. 2005).

In the present study, we identified polymorphisms that have not yet been described in the huntingtin interacting proteins HIP1 and ZDHHC17 (HIP14), and investigated their role as modifiers of age-at-onset in HD. We also investigated polymorphisms in the genes of the glutamate receptor GRIK2, the TATA binding protein (TBP) and the brain-derived neurotrophic factor (BDNF), that are already known to be associated with HD, in a large group of more than 900 European HD patients.

Materials and methods

HD patients

A total of 980 unrelated European HD patients was investigated. Of these, 383 patients were of German descent, and 341 patients were of Italian descent. The remaining 256 patients were from other European countries. All subjects gave informed consent according to the declaration of Helsinki. For all patients, HD was clinically diagnosed and the age-at-onset was estimated as the age when motor or cognitive symp-

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toms were first noticed. The age-at-onset ranged from 5 to 85 years with a mean age-at-onset of 45.1 years (SD 13.4). CAG repeat lengths in the *HD* gene had been tested in all patients. Repeat numbers of patients deriving from other European institutions were randomly checked in our laboratory with a reference control. The number of the expanded allele ranged from 39 to 90 repeats. The median repeat number was 44.

Mutation analysis and genotyping of *ZDHHC17* and *HIP1*

PCR amplification that preceded the mutation analysis was performed according to standard conditions with 50–150 ng DNA, 0.4 μM of each primer, 1 × PCR buffer (Genecraft, Germany), 200 μM of each dNTP, 10% Q-Solution (Qiagen, Germany); for *ZDHHC17* g.-886A > C and g.-844G > T) and 1U Taq polymerase (Genecraft) under following conditions: 2′ 96°C, (1′ 96°C; 1′ annealing temperature; 1′ 72°C) 35 cycles, 10′ 72°C, 4°C ∞.

To screen for unknown sequence variations in the *ZDHHC17* and *HIP1* genes in 60 control samples from the Centre d'Etude du Polymorphisme Human (CEPH) cohort, we used denaturing high-performance liquid chromatography (dHPLC). We analysed all exons and the promoter region of the *ZDHHC17* gene and the huntingtin binding sites in *HIP1* (exons 3–13). The position of the promoter was proposed by ElDorado (Genomatix Software GmbH, Munich, Germany) http://www.genomatix.de/cgi-bin/eldorado-fr31. For detecting sequence changes, PCR products were analysed on a Wave® DNA Fragment Analysis system (Transgenomic, Inc., San Jose, CA, USA). The specific column temperatures for *ZDHHC17* and *HIP1* are shown in Table 1. Aberrant peak patterns indicating changes in the DNA sequence were collected, purified using a PCR purification kit (Qiagen) and sequenced by CEQ 8000 Dye Terminator Cycle Sequencing (Beckman Coulter, Inc., Fullerton, CA, USA) with the same primers used for the PCR. Genotyping of the *ZDHHC17* polymorphisms g.-866A > C and g.-844G > T was performed via dHPLC.

The polymorphism N384S in *ZDHHC17* was first amplified with new mismatch forward and reverse primers to allow restriction analysis. The mismatch primer was generated using dCaps Finder 2.0 software http://www.helix.wustl.edu/dcaps/dcaps.html. For restriction analysis, the PCR product was incubated with HindIII (3U) according to the manufacturer's instructions (New England Biolabs, Inc., Berverly; MA, USA). In the presence of the base pair change g.77751A > G, HindIII digested the 223 bp product into fragments of 197 bp and 26 bp.

Table 1 Sequence variations found in the genes encoding ZDHHC17 and HIP1

Gene	Sequence variation	Amino acid	Heterozygosity in CEPH control individuals
ZDHHC17[a]	g.41238–41242delGACTT		0.250
	g.58264G > A	P290P	0.250
	g.62940G > A		0.183
	g.77751A > G[c]	N384S	0.127
	g.84024C > T	Y513Y	0.016
	g.86625A > G	R591R	0.016
	g.-866A > C[c]		0.016
	g.-844G > T[c]		0.282
HIP1[b]	g.17151T > C	A50A	0.083
	g.25508C > T		0.083
	g.39605G > A		0.033

The nomenclature for numbering of changes at nucleotide or amino acid level is according to general rules (den Dunnen and Antonarakis 2001)

[a] Reference sequence AcNo. NC_000012

[b] Reference sequence AcNo. NC_000007

[c] Polymorphisms analysed in this study

Genotyping of huntingtin, GRIK2 and TBP

Polymorphic repeats in *huntingtin*, *GRIK2* and *TBP* were determined by PCR amplification and fragment length analysis. PCR conditions and the primer set for *huntingtin* were used as previously described (Riess et al. 1993). Amplification of the *GRIK2* repeat was modified according to Paschen et al. (1994). The analysis of the length of the CAG repeat in *TBP* was slightly modified to 5′ 95°C, (1′ 95°C; 2′ 60°C; 1,5′ 68°C) 32 cycles, 10′ 68°C, 4°C ∞ (Rolfs et al. 2003). For each gene two different labelled forward primers were used, respectively, so two samples could be pooled after PCR and measured in one approach. Determination of the repeats was carried out by fragment analysis with the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc.) according to the manufacturer's instructions.

Genotyping of BDNF

After standard amplification, the V66M polymorphism was detected by pyrosequencing with the PSQ 96MA system (Biotage AB, Uppsala, Sweden). Three other non-synonymous variations in BDNF (Q75H, R125M, R127L; published in dbSNP) have been screened in the 60 control samples (CEPH) by pyrosequencing, but none of them turned out to be polymorphic and thus were not analysed in our age-at-onset modifier study.

Statistical analyses

Allele and genotype frequency and Hardy–Weinberg distribution of genotypes were determined by Genepop version 3.3 (http://www.wbiomed.curtin.edu.au/genepop). A possible modifying effect on the HD age-at-onset of the respective polymorphisms was investigated by applying a model of analysis of variance by JMP® Version 5.1 (SAS institute, Inc., Cary, NC, USA). The goodness of fit was evaluated by the proportion of variation in the age-at-onset, explained by the coefficient of determination (R^2). We obtained the best fit of our data by logarithmic transformation of the age-at-onset and the CAG repeat number. For analysis, variance in the age-at-onset for the CAG repeats in huntingtin was determined alone, as well as in addition with different polymorphisms. A change of R^2 indicated a relative improvement of the model when the respective factors were added to the effect of the expanded huntingtin allele (ΔR^2). This identified the percentage of the variance that was attributable to the candidate modifier loci, when there was a significant P value. A P value of less than 0.05 was considered significant.

Depending on the respective polymorphisms, some patients could not be genotyped and they were therefore excluded from statistical analyses. Additional power analyses were performed to determine the minimum number of patients for minimizing the statistical error of type II.

Results

Screening for polymorphism in *ZDHHC17* and *HIP1*

Screening the genes encoding *ZDHHC17* and *HIP1* in 60 control samples (CEPH), we found several genetic variations (Table 2). Among the exonic variations we detected one non-synonymous change (N384S). The others were silent variations. The polymorphisms N384S, g.-886A > C and g.-844G > T were selected due to their potential functional relevance and their relative frequency. In *HIP1* one silent exonic variation and two intronic polymorphisms were detected (Table 1). HIP1 polymorphisms were not further investigated.

Analysis of potential age-at-onset modifier polymorphisms

To test the contribution of polymorphic changes on disease onset, the genotypes in *ZDHHC17* (N384S, g.-886A > C and g.-844G > T) and previously described polymorphisms in the *GRIK2* (TAA repeat), *TBP* (CAG repeat) and *BDNF* gene (V66M) were determined in 980 HD patients.

Table 2 Allele frequency of polymorphisms in genes encoding GluR6, TBP, BDNF and HIP14 and genotype frequency of nucleotide substitutions in genes encoding BDNF and HIP14

Gene	Polymorphism	Allele frequency[b]		
		1	*2*	*3*
GRIK2[b]	g.669992(TAA)9–17 (n = 966)	0.043	0.931	0.026
TBP[b]	g.4879(CAG)26–41 (n = 954)	0.049	0.903	0.048

		Allele frequency[c]		Genotype frequency		
		1	*2*	*1-1*	*1-2*	*2-2*
BDNF[a]	V66M (g.196G > A) (n = 954)	0.744	0.256	0.546	0.395	0.059
ZDHHC17[a]	N384S (g.77751A > G) (n = 965)	0.885	0.115	0.780	0.209	0.011
	g.-886A > C (n = 968)	0.990	0.010	0.979	0.021	–
	g.-844G > T (n = 968)	0.827	0.173	0.677	0.301	0.023

The nomenclature for numbering of changes at nucleotide or amino acid level is according to general rules (den Dunnen and Antonarakis 2001)

All genotypes observed did not differ from expectations under Hardy–Weinberg equilibrium (n = number of investigated persons whose genotype could be determined)

[a] Reference sequence AcNo. NC_000006 (GRIK2, TBP), Reference sequence AcNo. NC_000011 (BDNF), Reference sequence AcNo. NC_000012 (ZDHHC17)

[b] For determination of the allele frequency of GRIK2 and TBP to show a distribution pattern of the alleles CAG and TAA repeats were grouped concerning short, medium and long alleles. *GRIK2*: *1* 9–12 repeats, *2* 13–15 repeats, *3* 16–17 repeats; *TBP*: *1* 26–32 repeats, *2* 33–37 repeats, *3* 38–41 repeats. The arrangement was due to the distribution on Hardy–Weinberg in the respective groups. Genotype frequency of GRIK2 and TBP is not presented because of the quantity of genotypes

[c] Allele frequency of nucleotide substitutions in BDNF and ZDHHC17 is described by *1* wild type allele, *2* variant allele

For the TAA repeat near the 3′ region of the *GRIK2* gene we found nine alleles in HD patients. The TAA repeat length ranged from 9 to 17 repeats. The three most frequently detected alleles were those with 14, 15 and 13 TAA repeats, respectively. The longest repeat with 17 alleles was observed in about 0.5% of the samples, but it was not associated with a longer CAG repeat in huntingtin or a remarkable age-at-onset (data not shown). In the *TBP* gene a range of 26 to 41 CAG repeats were observed. The respective median repeat number was 36. The longest allele of 41 CAG repeats was found in only two HD patients without a remarkable low age-at-onset (data not shown).

Allele frequencies of both of the polymorphic repeats in *GRIK2* and *TBP* and the polymorphisms in the genes encoding BDNF and ZDHHC17 are listed in Table 2; the different alleles in GRIK2 and TBP are divided into three groups for a simplified demonstration. All genotypes observed did not differ from expectations under Hardy–Weinberg equilibrium. Concerning the three polymorphisms in the *ZDHHC17* gene, we could observe four haplotypes, but they did not appear to be good markers for HD. None of them are associated with an older or younger age-at-onset (data not shown).

Effect of polymorphisms on the age-at-onset

The significant effect of the expanded CAG repeat number in the *huntingtin* gene on the age-at-onset of HD patients has been shown in numerous studies (Rubinsztein et al. 1997; The U.S.–Venezuela Collaborative Research Project and Wexler 2004). In our study, we could also confirm this observation by applying a statistical model of an analysis of variance. The value of R^2 achieved 0.52 indicating that 52% of the variation in age-at-onset could be explained by the expanded CAG repeats. In addition to the number of the expanded CAG repeat in huntingtin, the modifying effects of the polymorphic repeats in *GRIK2* and *TBP* and the polymorphisms in *BDNF* (V66M) and *ZDHHC17* (N383S, g.-886A > C and g.-844G > T) on the age-at-onset were examined. Neither the examined polymorphisms nor the normal CAG repeat in huntingtin showed a significant effect on the age-at-onset of HD patients (Table 3). Also, testing for additive effects of different factors resulted in no significant effect (data not shown). Furthermore, we investigated patients of German and Italian ancestry separately in order to detect a specific effect in different populations. However, the respective polymorphisms showed no effect in each of these groups either (data not shown).

Discussion

In the present study, we characterized a large number of polymorphisms in genes that are suggested to act as possible modifiers for the age-at-onset of HD. Genetic modifier factors have been indicated in HD as the length of the disease causing expanded polyglutamine tract in huntingtin explains only 42–73% of the variance in the age-at-onset (Brinkman et al. 1997; Stine et al. 1993; The U.S.–Venezuela Collaborative Research Project and Wexler 2004). In our study, the polyglutamine repeat accounts for 52% of the variance in age-at-onset which is in good agreement with other studies which analysed the effect of the expanded CAG repeat on the age-at-onset.

Table 3 Analysis of variance of possible candidate genetic modifiers

Model	R^2	ΔR^2	*P* value	Least significant number of patients (power analysis)
HD CAG	0.5274	–	<0.0001	7
HD CAG + normal CAG (htt)	0.5289	0.0015	0.0861	1,279
HD CAG + GRIK2 genotype	0.5374	0.0100	0.7986	2,130
HD CAG + TBP genotype	0.5510	0.0236	0.4484	1,281
HD CAG + TBP CAG_{exp}	0.5276	0.0002	0.9226	4,243
HD CAG + BDNF V66M	0.5302	0.0028	0.6450	6,518
HD CAG + HIP14 N384S	0.5290	0.0016	0.3149	2,503
HD CAG + HIP14 g.-886A > C	0.5350	0.0076	0.7118	272,245
HD CAG + HIP14 g.-844G > T	0.5358	0.0084	0.3905	3,084

The nomenclature for numbering of changes at nucleotide or amino acid level is according to general rules (den Dunnen and Antonarakis 2001)

The level of significance was set to $P = 0.05$; $n = 980$

HD CAG expanded CAG allele in huntingtin; *htt* huntingtin; *TBP* CAG_{exp} longer allele in TBP

The evaluation of the age-at-onset presents a challenge that has to be solved as precise as possible. Mostly the accurate determination and temporal classification are up to the patients' relatives. Initial symptoms of HD are usually subtle and complex thus different studies often base their determination of the age-at-onset on the occurrence of different symptoms (Chattopadhyay et al. 2003; MacDonald et al. 1999). Recent studies concerning the cell loss in the affected brain regions showed that there is a correlation between the symptomatology in HD and predominant cell loss in different brain regions responsible for motor coordination or mood activities, respectively (Egan et al. 2003). Thus, an exact method determining the age-at-onset needs to recognize motor and cognitive symptoms. However, the most accurate age-at-onset data might only be achieved in prospective studies in genetically tested persons at risk.

Genome wide linkage analysis identified several chromosomal regions linked with the age-at-onset of HD. Measured by LOD scores the tip of chromosome 4 (4p16) and human chromosome 6 showed the highest linkage. The location of a genetic modifier, which is located near the *huntingtin* gene in 4p16, is also supported by other studies (Li et al. 2003; Thu et al. 2005).

On the other hand, whole genome linkage studies may only detect major modifier factors, which may not correspond to the natural situation in HD. Thus, association studies investigating polymorphisms of candidate genes are one of the options to identify these modifiers. Here we examined polymorphisms in the genes encoding GRIK2, TBP, BDNF, ZDHHC17 and HIP1, which directly interact with huntingtin protein or have been suggested as modifiers in previous studies. The most frequently analysed gene in this respect is the *GRIK2* gene. Previous studies demonstrated that a specific allele of a TAA repeat near the 3′ terminal of *GRIK2* is associated with a younger age-at-onset in HD (Chattopadhyay et al. 2003; MacDonald et al. 1999; Rubinsztein et al. 1997). The share of the *GRIK2* polymorphism in the variance of the age-at-onset differed widely among the three studies. The authors suggested that 2–13% of the variance, which was not accounted for by the CAG repeat in *huntingtin*, could be attributed to the genotype variation in *GRIK2*. However, in our sample we could not confirm this observation although our analysis contained with 980 patients a much larger collective than previous studies (70 to 300 HD affected patients, respectively). Besides the sample size, differences between the studies might be explained by the different ethnic origins of the patients. The patients of the previous studies were from English, Eastern American and Eastern Indian descent. In our group, which consisted predominantly of German and Italian patients, we did not find any effect of the *GRIK2* polymorphism in ethnically uniform subgroups. An additional power analysis showed that one would need at least 2,130 unrelated single patients to detect significant effects of *GRIK2* further indicating the limitations of all previous association analyses. Also, genome scans for modifiers for HD showed no definite evidence for linkage at 6q16.3-q21 on the chromosomal location of *GRIK2* (Li et al. 2003).

Several other studies have indicated an association of the *TBP* gene with HD (Djoussé et al. 2004). The *TBP* gene is a good candidate as the encoded protein forms insoluble aggregates in the nucleus of neuronal cells in HD patients (Djoussé et al. 2004). Similar to *huntingtin*, *TBP* contains a polymorphic CAG repeat, which ranges from 26 to 42 repeats in normal individuals. Mutant huntingtin interacts with TBP and impairs the functional conformation of the transcription factor (Yanagisawa et al. 2000); however, we found no indication that the CAG repeat length of the TBP does influence the age-at-onset in HD.

It has also been demonstrated that wild type huntingtin has anti-apoptotic properties. In fact, wild type huntingtin up-regulates transcription of the brain-derived neurotrophic factor (*BDNF*), which is produced by cortical neurons and acts as a pro-survival factor for neurons in the striatum (Schaffar et al. 2004). Huntingtin also enhances the transport of BDNF-containing vesicles along microtubules to striatal cells (Zuccato et al. 2001). In its mutant form huntingtin results in a decreased production and transport of BDNF. Though a recently published study determined an association of V66M with the age-at-onset of HD (Alberch et al. 2005), we could not detect a significant effect of V66M on the age-at-onset in our study.

We finally investigated the huntingtin interacting proteins HIP1 and ZDHHC17. ZDHHC17 is localized predominantly in brain and partially colocalized with huntingtin in the medium spiny neurons of the striatum (Singaraja et al. 2002). Since it contains several transmembrane and ankyrin repeat domains, ZDHHC17 is suggested to play a role in endocytosis and intracellular protein trafficking. Most interestingly, interaction of ZDHHC17 with huntingtin is inversely correlated to the polyglutamine length in huntingtin suggesting an impaired neuronal transport in HD (Singaraja et al. 2002). Like ZDHHC17, the interaction between HIP1 and huntingtin is inversely correlated to the polyglutamine length in huntingtin (Wanker et al. 1997). Because of its homology to Sla2p, HIP1 is suggested to be involved in cytoskeleton formation, vesicle transport and endocytosis. In the case of expanded huntingtin, its interaction with HIP1 is decreased and *HIP1* is

released. Through the binding of Hippi (HIP1 protein interactor) and the pDED of HIP1 the caspase-8-dependent apoptotic pathway is induced (Gervais et al. 2002). This process could explain different aspects of neuronal death in HD, which could be influenced by polymorphisms of HIP1.

While we did not find any polymorphisms in the N-terminal half of HIP1, which directly interacts with huntingtin (Wanker et al. 1997), we detected three polymorphisms in the *ZDHHC17* gene. As no region for the interaction of ZDHHC17 with huntingtin has been defined yet, we screened the entire gene for polymorphisms. We found one polymorphism in exon 11 (N384S) and two in the promoter region. None of them, however, added significantly to the age-at-onset in our patients.

It has also been discussed that environmental factors contribute to the variation in the age-at-onset in HD (The U.S.–Venezuela Collaborative Research Project and Wexler 2004). Recent evidence from a transgenic mouse model further supports this hypothesis. It was shown that environmental enrichment of transgenic R6/1 and R6/2 HD mice resulted in a delayed onset of motor symptoms and a delayed loss of cerebral volume (van Dellen et al. 2000; Hockley et al. 2002). The recent identification of the protective role of the disaccharide trehalose, which is a normal component of our nutrition, in HD mice further supports the role of environmental factors on the age-at-onset (Tanaka et al. 2004).

In conclusion, it is most likely that a whole network of genetic and environmental factors influences the age-at-onset in HD. Defining both environmental and genetic factors will be extremely important not only to gain more insights into the pathogenesis of HD, but also to more clearly define the effect of substances in future drug trials.

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