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RAPID COMMUNICATION

ASK1 and MAP2K6 as modifiers of age at onset in Huntington's disease

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Abstract Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative disease associated with abnormal expansions of a stretch of perfect CAG repeats in the *HD* gene. The number of repeat units is predictive for the age at onset (AO) of neurological symptoms. Part of the remaining variation in AO is attributed to modifier genes. In this study, genes involved in apoptosis were investigated as candidates for modulating AO in HD. A panel of 304 candidate genes was screened for allelic associations with motor AO via linked microsatellite markers by pooling the DNAs of HD individuals from opposite ends of the AO distribution. After genotyping promising markers from the pooling experiment individually, markers revealed consolidated evidence for association in a candidate region comprising the genes *MAP3K5* (*ASK1*)/*PEX7* at 6q23.3 and in the gene *MAP2K6* at 17q24.3. Fine-mapping of these candidate regions in a cohort of 250 Caucasian HD patients using single nucleotide polymorphism (SNP) markers delimitated the precise locations of association. Certain variations in an *ASK1–PEX7* haplotype block explain 2.6% of additional variance in AO in our HD cohort. In males, 4.9% additional variance could be attributed to *MAP2K6* genotype variations. Altogether, *ASK1–PEX7* haplotypes and *MAP2K2* genotype variations explain 6.3% additional variance in AO for HD. We hypothesise that sequence variations of *ASK1* and *MAP2K6* lead to partially sex-specific changes in the levels

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and/or phosphorylation states of p38 and p38-regulated proteins that might contribute to the observed delaying effects in the AO of HD.

Keywords Huntington's disease · Age at onset · Modifier genes · *MAP3K5* · *MAP2K6*

Introduction

Huntington's disease (HD [MIM 143100]) is an autosomal dominantly transmitted, progressive neurodegenerative disease associated with defined expansions in a stretch of CAG repeats in the 5′ part of the *HD* gene encoding the protein huntingtin (htt) [1]. Expanded CAG block lengths comprising 36–39 repeats may be associated with the clinical presentation of HD, repeat lengths of ≥40 units invariably give rise to clinical manifestation of HD [2]. The expanded CAG repeat is translated into an elongated poly glutamine (polyQ) tract, which apparently leads to neuronal dysfunction and neurodegeneration. Neuropathological features include dramatic loss of neurons and development of astrogliosis in the striatum [3], leading to choreiform movements potentially accompanied by psychiatric and cognitive disturbances. The length of the polyQ tract is the most critical factor for determining age at onset (AO) [4–6], albeit substantial variability remains after controlling for repeat length, particularly when expanded CAG blocks range in lengths ≤46 units [7, 8]. Individuals with identical repeat lengths may exhibit enormous variation in manifestation, progression and outcome of the disease possibly partly due to modifier genes and environmental factors impacting on disease presentation [8]. Defining these modifying genes may have major implications for understanding the pathogenesis. Yet, genetic factors underlying complex traits like AO are notoriously difficult to be identified because many genetic variations may contribute by having small individual effect and, in addition, because of incomplete penetrance. Hitherto, the association of motor AO has been reported with polymorphisms in nine genes [9]. Among these are variations in the NR2A and NR2B sub-units (encoded by *GRIN2A*, *GRIN2B*) of the NMDA-type glutamate receptor [10]. An intronic SNP in *GRIN2A* and the *GRIN2B* C2664T exchange confer significant effects on AO especially in female HD patients [11]. In addition to these candidate gene studies, evidence for the linkage of AO with the 6q23–24 region was confirmed in HD families [12, 13].

In this study, we pursued a strategy that involves pooling DNAs of patients from the opposite ends of the trait manifestation for determining the association between candidate markers and motor AO in HD. A host of results inter-link apoptotic pathways with the mutant htt protein, and models relating apoptosis to neuronal death in HD have been put forward to explain certain aspects of the pathogenesis [14]. Up-regulation of pro-apoptotic proteins may result in increased cleavage of htt [14], and subsequently arising smaller htt fragments have been proposed to cause cellular stress [15]. Furthermore, a component of the stress response that has been studied in HD represents the activation of mitogen-activated protein kinase (MAPK) signalling pathways [16], which control physiological processes and may

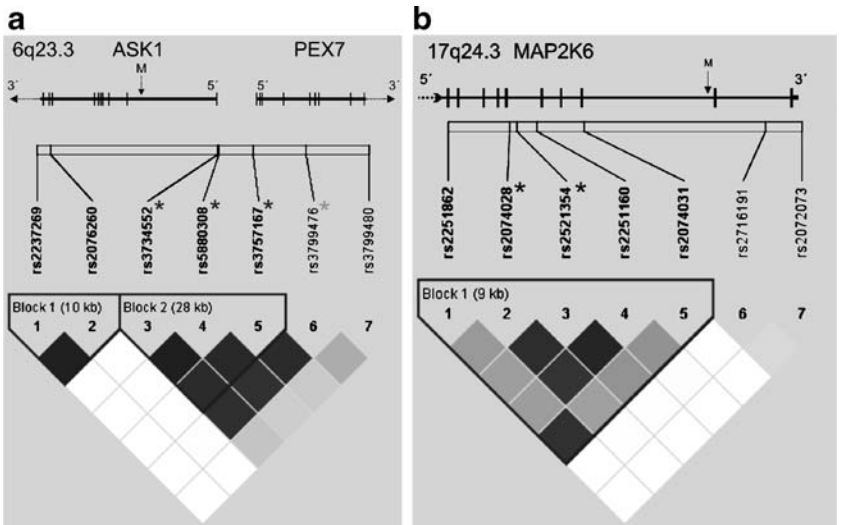

Fig. 1 Schematic representation of the **a** *ASK1–PEX7* and **b** *MAP2K6* gene region and pair-wise normalised linkage disequilibrium (LD) as calculated using the Haploview programme. *Vertical* and *horizontal lines* represent exons and introns, respectively. The *vertical black arrow* indicates the position of the originally investigated microsatellite marker (*M*). Haplotype blocks are *boxed*, comprised SNPs in *bold face*. Each square represents the magnitude of pair-wise LD. *Dark grey* shading indicates stronger pair-wise LD; *light grey* squares represent intermediate and *white squares* represent weak LD (R^2). *Asterisks* indicate SNPs showing significant association with AO

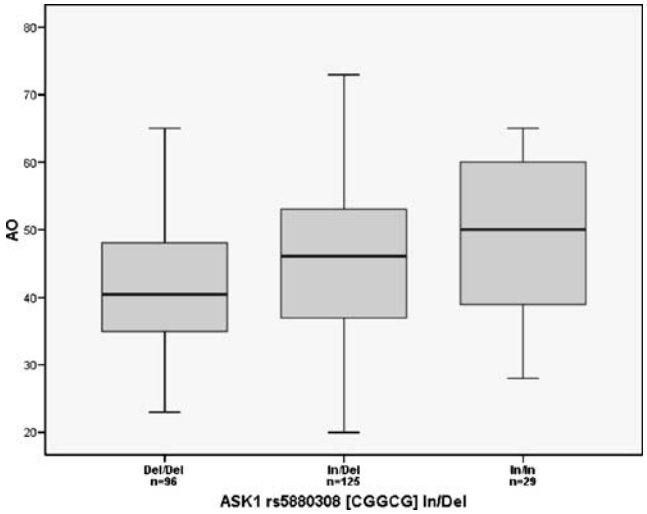

Fig. 2 Distributions of the *ASK1* (CGGCG) *In/Del* genotypes (rs5880308) and AO for 250 Huntington's disease patients

initiate apoptotic programmes. Hence, we screened a series of 304 micro-satellites mostly representing genes involved in these apoptosis-related pathways (see Table S1 of the electronic supplementary material).

Results

We screened 151 unrelated patients with clinical diagnosis of HD in an extended association screen with micro-satellite markers as described in detail before by using five DNA pools representing different AO intervals [17, 18; see also supplementary methods]. A total of 304 markers were investigated representing 304 genes involved in apoptosis (see Table S1 of the electronic supplementary material).

Markers for the c-*Jun* N-terminal kinase (JNK) pathway related genes *MAP3K5* (*ASK1*), *MAP2K6*, *MAPKAPK5* and *JUN*, and a marker for the cytokine gene *IL6* showed obvious inter-pool differences in allele image profiles (AIPs, see Fig. S1 of the electronic supplementary material). Individual genotyping and adjusting the variability for AO attributable to the CAG block length by linear regression confirmed the significant association of AO with markers for *ASK1* and *MAP2K6*.

MAP3K5 (ASK1): fine-mapping and re-screening

Genotyping additional SNPs spread across approximately 230 kb of the corresponding *ASK1* region (SNPs 1, 2, 3, 5, 6 and 7 in Fig. 1a, Fig. S3) in the entire HD patient cohort (n=250) verified the results of the initial screening and localised the association to a region in extensive LD (Fig. 1a) encompassing the inter-genic region between *ASK1* and *PEX7* and the 5′ regions of both genes. After screening 48 DNAs, re-sequencing of all deviant SSCP patterns revealed exclusively known SNPs without obvious functional consequences (see Table S2 of the electronic supplementary material). Yet the (CGGCG) *In/Del* poly-

Table 1 Linear regression analysis of polymorphisms in candidate genes affecting AO in HD

Model	R^2	ΔR^2	% Additional explained variance	P value
HD CAG	.577	–		<.0005
HD CAG+ASK1 rs5880308[a]	.587	.01	2.4	.010
HD CAG	.534	–		<.0005
Female HD CAG+ASK1 rs5880308	.542	.008	1.7	.085
HD CAG	.617	–		<.0005
Male HD CAG+ASK1 rs5880308	.626	.009	2.3	.050
HD CAG	.577	–	–	<.0005
HD CAG+MAP2K6 rs2521354[b]	.579	–	–	.138
HD CAG	.534	–	–	<.0005
Female HD CAG+MAP2K6 rs2521354	.530			.724
HD CAG	.617	–		<.0005
Male HD CAG+MAP2K6 rs2521354	.636	.019	4.9	.006
Male HD CAG+MAP2K6 rs2521354+ASK1 rs5880308	.641	.024	6.3	.013

The dependence of AO on CAG repeat block length in the *HD* gene was assessed by linear regression. The best fit estimated by the R^2 value was obtained after log transformation of AO. R^2 values illustrate the relative improvement of the regression model when the genotypes are considered in addition to the CAG repeat lengths; ΔR^2 values quantify these differences. CAG repeat lengths explain approximately 60% of the variance in AO. Multiple regression models were used to test the effect of different genotype variations. In these models, the genotype for each SNP was coded as dummy variable that represents the levels of a three-level categorical variable (additive genetic model) or two-level categorical variable, respectively. The addition of genotypes to the effect of CAG repeat lengths resulted in a significant increase of the R^2 value (0.577 to 0.587, p= 0.010). In *MAP2K6*, sex-specific addition of genotype variations to the effect of CAG repeat lengths resulted in an increase of the R^2 value only in males (0.617 to 0.626, p=0.050).

[a] Randomly chosen SNPs within the same haploblock: rs3734552, rs3757167 and rs3799476 yield the same results.

[b] Randomly chosen SNP rs2074028 within the same haploblock yields the same results.

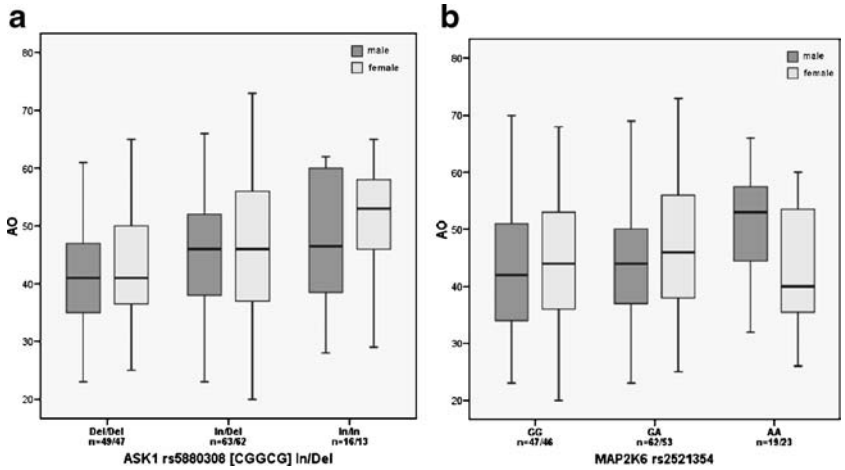

Fig. 3 Sex-specific distributions of the **a** *ASK1* (CGGCG) *In*/*Del* genotypes (rs5880308) and **b** *MAP2K6* rs2521354 genotypes and AO for 250 Huntington's disease patients. Box-plots of female (*light grey*) and male (*dark grey*) AO are presented for each genotype, boxes span the inter-quartile range from first to third quartile with a *horizontal line* showing the median; the range is also indicated by the *horizontal lines*

morphism is situated in the core promoter of *ASK1* (rs5880308, SNP4 in Fig. 1a). Furthermore, rs1474988 and rs5880308 are located in the region of the hypothetical *LOC7322745* gene with unknown function. But because the latter resides in the intron and rs1474988 is a synonymous polymorphism of apparently no functional significance, we mainly focused on *ASK1* and *PEX7*.

MAP2K6 fine-mapping

Genotyping seven additional SNPs (Fig. 1b, Fig. S3) in the expanded HD cohort revealed 5′ of the originally associated micro-satellite as a haplotype block comprising SNPs in intron 7 of the *MAP2K6* gene, rs2521354 and rs2074028, which are strongly associated with AO in HD patients (Fig. 1b).

AO-modifying effect

The SNPs in extensive LD in the inter-genic region between *ASK1* and *PEX7* show strong correlation with AO in HD as demonstrated in the box plot of the (CGGCG) *In*/*Del* genotypes (rs5880308, Fig. 2). Adding the genotypes to the effect of CAG block lengths resulted in significant increase of the R^2 value (0.577 to 0.587, p= 0.010, Table 1). Stratification of patients according to sex revealed no sex-specific differences (Fig. 3a).

Figure 3b depicts the box plots for the AO in the cohort stratified by the significantly associated *MAP2K6* rs2521354 SNP genotypes and sex. A genotype-dependent effect on AO is obvious in males where the AA genotype is associated with later AO. Controlling this effect by linear regression confirmed the correlation using the logarithmically transformed AO as the dependent variable and rs2521354 SNP genotypes as independent variables. Sex-specific addition of genotype variations to the effect of CAG repeat lengths resulted in an increased R^2 value only in males (0.617 to 0.626, p=0.050). A total of 4.9% additional variance was attributed to the rs2521354 genotype variations in males. Together, *ASK1* and *MAP2K6* genotype variations explain 6.3% additional variance in AO for HD in males. When including the previously published

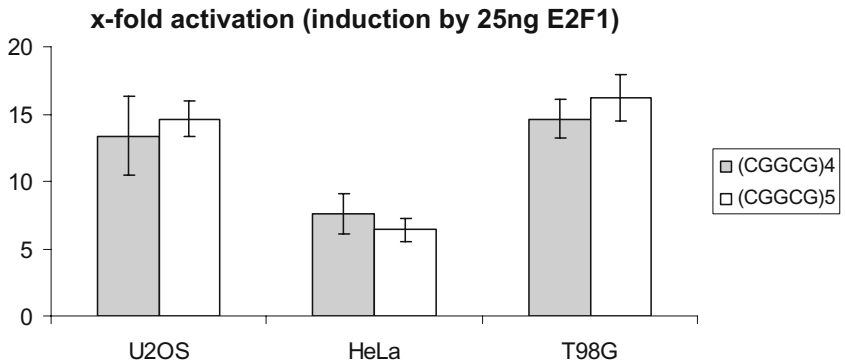

Fig. 4 Comparison of the responsiveness of the ASK1-$(CGGCG)_5$ and ASK1-$(CGGCG)_4$ promoter constructs to E2F1 in U2OS, HeLa and T98G cell lines. Results are expressed as "x-fold" increase over the pcDNA3-HA control expression (see supplementary methods)

GRIN2A and *GRIN2B* genotype variations [11], 5% additional variance in AO is explained for the total cohort, and 6% and 7.7%, for males and females, respectively.

Real-time PCR and reporter gene analyses

In transient transfection assays in the human cell lines with constructs expressing the ASK1-$(CGGCG)_5$ and ASK1-$(CGGCG)_4$ alleles of SNP4 and vectors expressing E2F1, no significant differences were observed in reporter gene activity (Fig. 4). Similarly, no differences were evident in *ASK1* or *PEX7* gene expression comparing individuals with common haplotypes (data not shown).

Discussion

Our results demonstrate significant evidence of association of variations in a genomic region comprising *ASK1* and *PEX7* at 6q23.3, a replicated linkage region for AO in HD [12, 13] and for *MAP2K6* at 17q24.3, respectively. The region at 6q23.3 comprises a haplotype block of high LD spanning *ASK1* (*MAP3K5*) and *PEX7* located 30 kb apart in head to head orientation. Peroxisomal assembly proteins encoded by *PEX* genes are required for import of matrix proteins into peroxisomes. Refsum disease, a peroxisomal disorder of branched chain lipid metabolism, is caused by *PEX7* mutations [19]. MAPKs are involved in a cascade of three sequentially phosphorylated and, hence, activated kinases [20]. Apoptosis signal-regulating kinase 1 (ASK1) is a MAPK kinase kinase (MAP3K) that is activated by various types of stress such as reactive oxygen species (ROS), endoplasmatic reticulum (ER) stress, calcium influx and immunological stress. ASK1 selectively activates two different sub-groups of MAP kinase kinases (MAPKK), SEK1 (MKK4) and MKK3/MAPKK6 (MKK6, encoded by *MAP2K6*), which in turn activate stress-activated protein kinase (SAPK, also known as JNK; c-*Jun* amino-terminal kinase) and p38 sub-groups of MAP kinases, respectively [21]. Different MAPKs were shown to play a role in HD: elongated htt activates JNK in HN33 hippocampal neuronal cell lines whilst wild-type htt did not [22]. ER stress-triggered by proteasomal dysfunction activates ASK1 via the formation of an IRE1–TRAF2–ASK1 complex. Furthermore, $ASK1^{-/-}$ primary neurons are defective in polyQ-, proteasome inhibitor- and ER stress-induced JNK activation and cell death [23]. These findings suggest that ASK1 is a key element in ER stress-induced cell death that plays an important role in the neuropathological alterations in polyQ diseases, which in turn renders it a prime candidate gene in this region. Both ASK1 and MAP2K6 are upstream activators of the p38 signaling pathway. ASK1 directly phosphorylates and activates MAP2K6. We hypothesise that sequence variations of *ASK1* and *MAP2K6* lead to changes in the levels and/or phosphorylation states of p38 and p38-regulated proteins in ways that contribute to the observed delaying effects in motor AO of HD.

The (CGGCG) *In*/*Del* genotype, however, is not predicted to exert potential allelic differences in *cis*-acting regulatory function in transcription by the Matinspector database (http://www.genomatix.de) [24]. Recent studies show that *ASK1* is directly regulated by E2F1 and that the DNA-binding domain and the trans-activation domain of E2F1 are necessary for efficient E2F1 trans-activation of the ASK1 promoter [25]. Yet, quantitative gene expression analysis using reporter gene analysis and real-time PCR do not show any differences in expression.

Although intronic SNP rs2521354 in *MAP2K6* may cause altered splicing, we cannot infer from the currently available data that this is a functional locus. Nevertheless, a recent study showed sex-related differences in p38 MAP kinase in mice after trauma and haemorrhage, the effect was dependent on testosterone and estradiol exposure [26]. Furthermore, gender-specific differences in p38 MAP kinase production have been observed in rodent models of myocardial ischaemia and reperfusion [27].

In summary, we report a specific *ASK1–PEX7* haplotype block within the 6q23–24 region that may harbour biologically significant variations modifying AO in HD. Uncovering *MAP2K6* as a putative modifier gene in HD highlights the potential role of the p38 signalling pathway as a key regulatory component in HD pathogenesis. Although our findings are preliminary and need to be replicated in a larger cohort, the results add to the evidence that, besides the *HD* mutation itself, alterations of other cell pathways may contribute to the inherited variability of the HD phenotype. Sex should be taken into account when modelling for modifier genes in HD as suggested previously.

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References

1. The Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983
2. Wieczorek S, Epplen JT (2006) Huntington's disease. In: Ganten D, Ruckpaul K (eds) Encyclopedic reference of genomics and proteomics in molecular medicine. Springer, Berlin
3. Myers RH, Vonsattel JP, Paskevich PA, Kiely DK, Stevens TJ, Cupples LA, Richardson EP, Bird ED (1991) Decreased neuronal and increased oligodendroglial densities in Huntington's disease caudate nucleus. J Neuropathol Exp Neurol 50:729–742
4. Snell RG, MacMillan JC, Cheadle JP, Fenton I, Lazarou LP, Davies P, MacDonald ME, Gusella JF, Harper PS, Shaw DJ (1993) Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. Nat Genet 4:393–397

5. Andrew SE, Goldberg YP, Kremer B, Telenius H, Theilmann J, Adam S, Starr E, Squitieri F, Lin B, Kalchman MA et al (1993) The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. Nat Genet 4:398–403
6. Duyao M, Ambrose C, Myers R, Novelletto A, Persichetti F, Frontali M, Folstein S, Ross C, Franz M, Abbott M et al (1993) Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat Genet 4:387–392
7. Kehoe P, Krawczak M, Harper PS, Owen MJ, Jones AL (1999) Age of onset in Huntington disease: sex specific influence of apolipoprotein E genotype and normal CAG repeat length. J Med Genet 36:108–111
8. The U.S.–Venezuela Collaborative Research Project, Wexler NS (2004) Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. Proc Natl Acad Sci U S A 101:3498–3503
9. Andresen JM, Gayan J, Cherny SS, Brocklebank D, Alkorta-Aranburu G, Addis EA, Cardon LR, Housman DE, Wexler NS (2007) Replication of twelve association studies for Huntington's disease residual age of onset in large Venezuelan kindreds. J Med Genet 44:44–50
10. Arning L, Kraus PH, Valentin S, Saft C, Epplen JT (2005) NR2A and NR2B receptor gene variations modify age at onset in Huntington disease. Neurogenetics 6:25–28
11. Arning L, Saft C, Wieczorek, S, Andrich, J, Kraus PH, Epplen JT (2007) NR2A and NR2B receptor gene variations modify age at onset in Huntington disease in a sex-specific manner. Hum Genet 122:175–182
12. Li JL, Hayden MR, Almqvist EW, Brinkman RR, Durr A, Dode C, Morrison PJ, Suchowersky O, Ross CA, Margolis RL, Rosenblatt A, Gomez-Tortosa E, Cabrero DM, Novelletto A, Frontali M, Nance M, Trent RJ, McCusker E, Jones R, Paulsen JS, Harrison M, Zanko A, Abramson RK, Russ AL, Knowlton B, Djousse L, Mysore JS, Tariot S, Gusella MF, Wheeler VC, Atwood LD, Cupples LA, Saint-Hilaire M, Cha JH, Hersch SM, Koroshetz WJ, Gusella JF, MacDonald ME, Myers RH (2003) A genome scan for modifiers of age at onset in Huntington disease: The HD MAPS study. Am J Hum Genet 73:682–687
13. Li JL, Hayden MR, Warby SC, Durr A, Morrison PJ, Nance M, Ross CA, Margolis RL, Rosenblatt A, Squitieri F, Frati L, Gomez-Tortosa E, Garcia CA, Suchowersky O, Klimek ML, Trent RJ, McCusker E, Novelletto A, Frontali M, Paulsen JS, Jones R, Ashizawa T, Lazzarini A, Wheeler VC, Prakash R, Xu G, Djousse L, Mysore JS, Gillis T, Hakky M, Cupples LA, Saint-Hilaire MH, Cha JH, Hersch SM, Penney JB, Harrison MB, Perlman SL, Zanko A, Abramson RK, Lechich AJ, Duckett A, Marder K, Conneally PM, Gusella JF, MacDonald ME, Myers RH (2006) Genome-wide significance for a modifier of age at neurological onset in Huntington's disease at 6q23–24: the HD MAPS study. BMC Med Genet 7:71
14. Hickey MA, Chesselet MF (2003) Apoptosis in Huntington's disease. Prog Neuro-psychopharmacol Biol Psychiatry 2:255–265
15. Kim YJ, Yi Y, Sapp E, Wang Y, Cuiffo B, Kegel KB, Qin ZH, Aronin N, DiFiglia M (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's dise brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci U S A 22:12784–12789
16. Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911–1912
17. Jagiello P, Gencik M, Arning L, Wieczorek S, Kunstmann E, Csernok E, Gross WL, Epplen JT (2004) New genomic region for Wegener's granulomatosis as revealed by an extended association screen with 202 apoptosis-related genes. Hum Genet 114:468–477
18. Wieczorek S, Jagiello P, Arning L, Dahmen N, Epplen JT (2004) Screening for candidate gene regions in narcolepsy using a microsatellite based approach and pooled DNA. J Mol Med 82:696–705
19. Braverman N, Chen L, Lin P, Obie C, Steel G, Douglas P, Chakraborty PK, Clarke JT, Boneh A, Moser A, Moser H, Valle D (2002) Mutation analysis of PEX7 in 60 probands with rhizomelic chondrodysplasia punctata and functional correlations of genotype with phenotype. Human Mutat 20:284–297
20. Weston CR, Lambright DG, Davis RJ (2002) Signal transduction. MAP kinase signaling specificity. Science 296:2345–2347
21. Nagai H, Noguchi T, Takeda K, Ichijo H (2007) Pathophysiological roles of ASK1-MAP kinase signaling pathways. J Biochem Mol Biol 40:1–6
22. Liu YF (1998) Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. J Biol Chem 273:28873–28877
23. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 16:1345–1355
24. Werner T (2000) Computer-assisted analysis of transcription control regions. Matinspector and other programs. Methods Mol Biol 132:337–349
25. Kherrouche Z, Blais A, Ferreira E, De Launoit Y, Monté D (2006) ASK-1 (apoptosis signal-regulating kinase 1) is a direct E2F target gene. Biochem J 396:547–556
26. Angele MK, Nitsch S, Knoferl MW, Ayala A, Angele P, Schildberg FW, Jauch KW, Chaudry IH (2003) Sex-specific p38 MAP kinase activation following trauma-hemorrhage: involvement of testosterone and estradiol. Am J Physiol Endocrinol Metab 285:189–196
27. Imahara SD, Jelacic S, Junker CE, O, Keefe GE (2005) The influence of gender on human innate immunity. Surgery 138:275–282