Age at onset in Huntington’s disease and methylation at D4S95

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Abstract
Age at onset in Huntington’s disease (HD) is variable and is influenced by parental sex, paternal age, and genetic background. Several recent models have tried to explain this variable expressivity by invoking parental imprinting and related aspects of epigenetic inheritance. Some of these mechanisms may result in variable DNA methylation at or near the HD gene. We show here that methylation at D4S95, a locus tightly linked to the HD gene, is highly variable. A comparison between patients with early onset HD, late onset HD, and normal controls showed no significant correlation between methylation and age at onset. However, we found a significant association of the age of the patient with demethylation at D4S95. Older persons tend to have lower levels of methylation at this locus. This observation is of interest with regard to studies that show an effect of paternal age, or more generally of ‘ageing genes’, age at onset in HD.

Huntington’s disease is a fully dominant and virtually fully penetrant neurodegenerative disorder. While the underlying biochemical defect is unknown, the HD gene has been localised to the short arm of chromosome 4. Tightly linked DNA markers are available and are currently being used in predictive testing. The rate of new mutations is extremely low, and the detection of linkage disequilibrium with close markers such as D4S95 is consistent with the hypothesis that very few separate mutations may account for the worldwide distribution of HD.

While the HD gene seems to be fully penetrant there is very wide variation in the expressivity of the gene as witnessed by the age of the patient at which symptoms start. Age at onset in the patient is influenced by at least three factors. First, age at onset is more similar within families than between families, suggesting that background genes, or modifiers, influence the expressivity of the HD gene. Second, expressivity is affected by the sex of the transmitting parent, such that on average earlier onset is produced with paternal inheritance. Recent work has conclusively shown that paternal transmission cases fall into two groups, one in which the onset is only slightly, but significantly, younger than with maternal transmission, and the other, much smaller group in which onset is dramatically (average 24 years) younger than in the parent (major anticipation). It was also shown that in this group, which constitutes about 6% of all paternal transmission cases, rigidity as a symptom is associated with very early onset, major anticipation, and generally young age at onset in the parent. A third factor that influences age at onset is paternal age, with older fathers tending to have offspring with earlier onset of the disease.

A number of authors have suggested that this pattern of inheritance can be explained by epigenomic imprinting, by which the HD gene could be epigenetically modified according to the sex of the transmitting parent. Thus, modification of paternal origin would lead to earlier or higher level expression of the mutant gene. However, because paternal transmission cases fall into two categories, paternal imprinting has to be variable. This variability has been variously ascribed to rare modifying alleles on the X chromosome, or to stochastic or accidental variation in paternal imprinting; there is no general agreement at present as to the precise mechanism involved.

Work in the mouse has shown that imprinting of transgenes, and perhaps also of endogenous genes, is associated with DNA methylation, although other epigenetic modifications may also play a role. Thus, imprinted transgenes generally show less methylation when paternally inherited and hypermethylation when maternally derived. To begin to test some of the hypotheses on age at onset and imprinting, it would be desirable to analyse methylation patterns of the HD gene, especially in early onset versus late onset cases. This is at present not possible. However, we were encouraged to see that in two recent studies variable methylation was observed at D4S95, a locus that shows very tight linkage to the HD gene. Here we ask the question whether or not methylation at D4S95 is correlated with age at onset of HD.

Methods
DNA was extracted from peripheral blood according to standard methods. DNA samples (15 μg) were digested with the methylation sensitive restriction enzyme HpaII under conditions recommended by the supplier (NBL). To ensure complete digestion, a large excess of enzyme was used. Completeness was monitored by inclusion of phage λ DNA in the digest followed by electrophoresis of aliquots, ethidium bromide staining, and visual inspection of digestion products under UV illumination. Digested samples were electrophoresed on 0.8% agarose gels, blotted onto Hybond N membranes (Amersham), and hybridised with
the insert of pBS674 (D4S95) and with YGA2 oligolabelled with $^{32}$P dCTP. Hybridisation and washing were by standard procedures. Autoradiographic exposures that were within the linear range of the film were scanned on a Joyce-Loeblchromoscan. The methylation index of each sample was expressed as the ratio of intensity of the major digestion product versus that of the undigested fraction.

The statistical significance of intergroup differences in methylation index was assessed with the Mann–Whitney U test. The correlation coefficient between methylation index and age at blood sampling was calculated by the Spearman rank correlation coefficient (R). Statistical significance was taken at the 5% level.

Results
We decided to compare methylation at D4S95 in three different groups of subjects. The first was a group of 25 unrelated normal controls with a median age at blood sampling of 36 years (range 7 to 77 years). The second was a group of 13 patients from 12 kindreds with early onset HD (age at onset < 30 years, median age at onset 22 years, range 3 to 28 years); four of these had a rigid phenotype. The third was a group of 18 unrelated patients with late onset HD (age at onset > 50 years). In all cases, DNA was analysed from peripheral blood, and not from lymphoblastoid cell lines, because methylation is known to change in cultured cells. Methylation at D4S95 was determined by digestion with the methylation sensitive enzyme HpaII and hybridisation with the D4S95 probe. A major digestion product is produced at 7 kb, the intensity of which is variable in comparison with the undigested fraction at the top of the gel (fig 1). This suggests that methylation at HpaII sites near the D4S95 locus varies from person to person. All gels were scanned and the methylation index was expressed as the ratio of the intensity of the major digestion product versus the undigested fraction.

A higher methylation index thus indicates lower methylation at D4S95. Very considerable variation in methylation was found between subjects: the highest methylation index was 2.037, whereas the lowest one was 0.071, and the overall variation is therefore more than 28 fold (fig 2).

We next compared the methylation indices for subjects in each of the three study groups and this comparison is shown in fig 2. While the mean methylation index in the early onset HD patients (0.401, SD 0.413, median 0.34, n = 13) was lower than that of the late onset HD patients (0.528, SD 0.248, median 0.49, n = 18) and that of the controls (0.553, SD 0.426, median 0.48, n = 25), none of the intergroup differences reached statistical significance (early onset v late onset U = 70, p > 0.05; early onset v control U = 123, p > 0.05; late onset v control U = 198, p > 0.05). It also appears that there is no consistent intrafamily correlation between methylation status at D4S95 and age at onset in the early onset group. For example, the two subjects whose methylation analysis is shown in fig 1 are a pair of sibs whose age at onset is very similar (brother 23 years, sister 25 years) but whose methylation index differs more than four fold (brother 0.355, sister 0.071).

Because younger onset patients tend to be younger at the time of blood sampling than later onset ones, we also considered the possibility that variation in methylation at D4S95 was age related. Age at blood sampling was therefore compared with the methylation index in all patients and control subjects. Surprisingly, a significant correlation was found between age at sampling and methylation index (R = 0.489, df = 54, $t = 4.12$, $p < 0.001$), with older subjects showing a reduced level of methylation at D4S95 (fig 3). The lower average methylation index in the early onset group may thus be explained by an earlier age at sampling. A significant association of age at

Figure 1 (A) Methylation at D4S95 in control and Huntington's disease patients. a and b are two controls, c and d are two sibs with HD. Age at onset is 25 years for c and 23 years for d. Methylation indices are: a 1.46, b 1.08, c 0.07, d 0.36. (B) Methylation at YGA2t in subjects a, b, and c.

Figure 2 Methylation index in the early onset, late onset, and control groups. Each mark represents one subject. Note that a higher methylation index signifies lower levels of methylation.
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sampling and methylation index was also found in the control group on its own ($R^2 = 0.713$, $df=23$, $r = 4.88$, $p < 0.001$).

To see whether variable and age related methylation is a general feature of loci in the HD region on chromosome 4, we determined methylation at YGA2t, a probe located approximately 200 kb telomeric from D4S95.22 On digestion with HpaII, a band was produced at 0.9 kb whose intensity in relation to the undigested fraction was constant in different subjects (fig 1). There was no difference in methylation between HD patients and controls (fig 1 and data not shown).

Discussion
In this study a surprising level of variability of methylation in DNA from peripheral blood was found at a locus (D4S95) closely linked to the Huntington’s disease gene. The variation found between subjects of our study group was more than 28 fold. Methylation analysis of a variety of sequences in the human genome has shown that overall there is a very high degree of interperson concordance,23 although some sequences show differences between persons.24 Variability thus seems to be the exception rather than the rule. Indeed, another probe (YGA2t) in the HD region analysed in this study did not show any variability in methylation. We find that the major source of variability at D4S95 is age of the subject, and that with increasing age methylation at this locus decreases. Whether or not the remaining variability is genetically determined remains to be seen in appropriate family studies. Genetic factors can clearly play a role in determining methylation at variably methylated loci; such factors include alleles at the locus under study as well as alleles at unlinked loci.18

Several recent studies suggest the possibility that expressivity of the HD gene, and hence age at onset, might be associated with the degree of DNA methylation at or near the gene.9,11,15,16 Any variability of methylation observed near the HD gene is therefore of interest and begs the question of whether or not this variability is associated with age at onset. In this study we found that there is a tendency for early onset patients to have higher levels of methylation at D4S95 than late onset patients and subjects from the control group. This tendency, however, may be explained by the age related demethylation at this locus. Hayden et al (personal communication) have also investigated methylation status at a number of loci near the HD gene and have failed to find a significant association with age at onset. If there is an association of methylation with age at onset, it may well be confined to a small region in the vicinity of the gene, or indeed to specific sites within the gene.

The significance of the demethylation at D4S95 with age is at present not clear. There seems to be a general tendency to lose methyl groups in DNA with age,25,26 and this observation may explain the reactivation of an X chromosome linked gene in older female mice.27 It is not known, however, how general this loss of methylation is with respect to different gene sequences and different tissues. As far as we are aware this is the first study in which a specific DNA sequence has been found to undergo age related demethylation. Telomeres are known to shorten with senescence, and this could exert position effects on chromatin structure and methylation of telomere-near sequences28 (such as the HD region). It is interesting to note that two different influences of age on onset phenotype have been observed in HD. First, a general influence of ageing genes has been proposed to explain the association of longevity and age at onset in some families.29 Second, an effect of paternal age has been shown, with older fathers producing earlier disease onset in their children.14 This effect has been explicitly attributed to the loss of methylation during stem cell division in male gametogenesis.30 However, loss of methylation does not appear to be a general feature of loci in the HD region, as methylation at YGA2t, which is only 200 kb away from D4S95, was not variable. It will be interesting to see whether other sequences in this region, and in particular the HD gene itself, show age related or onset related methylation patterns.

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association between alleles detected at D4S95 and D4S96.