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Epigenetic regulation in amyloid precursor protein and the Lesch-Nyhan syndrome

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ABSTRACT

Lesch-Nyhan syndrome (LNS) is a neurogenetic disorder of purine metabolism in which the enzyme, hypoxanthine–guanine phosphoribosyltransferase (HPRT) is defective. A major unsolved question is how the loss of HPRT enzyme function affects the brain to cause the neurobehavioural syndrome in LNS and its attenuated variants (LNVs). To address this issue, a search for a link between LNS and the amyloid precursor protein (APP) is developed. Here, I identified, for the first time in fibroblasts from normal subjects as well as from LNS and LNV patients: (a) several APP-mRNA isoforms encoding divers APP protein isoforms ranging from 120 to 770 amino acids (with or without mutations and/or deletions) accounted for epigenetic mechanisms in the regulation of alternative APP pre-mRNA splicing and (b) five novel independent polymorphisms in the APP promoter: −956A>G, −1023T>C, −1161A>G, −2224G>A, −2335C>T relative to the transcription start site. A role for epistasis between mutated HPRT and APP genes affecting the regulation of alternative APP pre-mRNA splicing in LNS is suggested. An accurate quantification of various APP isoforms in brain tissues for detection of initial pathological changes or pathology development is needed. My findings may provide new directions not only for investigating the role of APP in neuropathology associated with HPRT-deficiency in LNS but also for the research in neurodevelopmental and neurodegenerative disorders by which various APP isoforms involved in the pathogenesis of the diseases such as Alzheimer's disease.

1. Introduction

Lesch-Nyhan syndrome (LNS) is a neurogenetic disorder of purine metabolism in which the enzyme, hypoxanthine–guanine phosphoribosyltransferase (HPRT, EC. 2.4.2.8; MIM 308000)), is defective [1,2]. The etiology involves a mutation of the HPRT gene, which is on the long arm of the X chromosome (Xq26.1), and it contains 9 exons and 8 introns [3]. Because the HPRT gene is on the X-chromosome, males are affected and females in the families are at risk of being carriers of the mutation. Complete or severe deficiency of HPRT activity leads to LNS (MIM 300322) [1]. Classical features of LNS include hyperuricemia and its sequelae (gout, nephrolithiasis, and tophi), motor disability (dystonia, chorea, and spasticity), intellectual impairment, and self-injurious behaviour. Partial deficiency of HPRT activity (MIM 300323) is characterized by the consequences of overproduction of uric acid and a variable spectrum of neurological manifestations, without the manifestations of self-injurious behaviour. The patients with partial deficiencies have been described as Lesch-Nyhan variants (LNVs) [4]. A major unsolved question is how the loss of HPRT enzyme function affects the brain to cause the neurobehavioural syndrome in LNS and its attenuated variants. To address this issue, a link between LNS and the aberrant basal ganglia function, including the dysfunction of dopaminergic pathways, was reported [5,6]. However, the mechanism by which features of LNS result from impaired purine metabolism is still not well understood. It was also documented that adhesion of HPRT-deficient neuroblastoma as well as fibroblasts from patients with LNS exhibited dramatically enhanced adhesion compared to control cells [7] and could have consequences for the maturation of the central nervous system, as seen in the smaller brain size of LNS and LNVs children [8,9]. Furthermore, it was also reported that HPRT deficiency was accompanied by aberrations in a variety of pathways know to regulate neurogenesis or to be implicated in neurodegenerative

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disease, including the canonical Wnt/β-catenin and the Alzheimer's disease/presenilin signaling pathways [10]. A role for the amyloid precursor protein (APP) relates to cell–cell or cell-substrate adhesion and is important for brain morphology and highly coordinated brain functions such as memory and learning has been suggested [11,12]. Hence, the APP pathway is possibly implicated in the development of LNS.

In an attempt to search for a link between LNS and APP, I have examined the APP-mRNA profile, the genomic APP-DNA, as well as the APP 5′ regulatory region in fibroblasts from four normal subjects and in HPRT-deficient fibroblasts derived from nine patients with LNS and three patients with LNV.

2. Materials and methods

2.1. Patients

This study includes four normal subjects, controls (samples # 1–4), nine LNS affected male patients (samples # 5–13), and three LNV affected male patients (samples # 14–16).

2.2. Isolations of genomic DNA, mRNA, amplifications, and cloning

RNA-free genomic DNA, and mRNA were separately isolated from intact cultured fibroblasts. For genomic DNA isolation, the Puregene®DNA Purification Kit (Gentra System, Minneapolis, Minnesota, U.S.A.) was used. For mRNA isolation, the FastTract®2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA, U.S.A.) was used. The DNA and mRNA concentrations were determined by using the ND-1000 spectrophotometer NanoDrop® device. For genomic characterization of the nine exons of the human HPRT gene locus and HPRT enzyme assay, I used the PCR primers, reagents and HPRT enzyme assay conditions as described in the previous work [13]. For genomic characterization of the eighteen exons of the human APP gene locus (GenBank NG_007376), sequence analysis of the APP 5′ regulatory region lying between −2693 and +296 (nucleotides 2355–5343) of the proximal (including exon 1) and distal APP promoter relative to the transcription start site (TSS, with +1 as nucleotide A of the TSS at 5048 in GenBank NG_007376), the PCR technique was used.

From the isolated mRNA, a first strand of cDNA was generated by means of reverse transcription (RT) using the reagents (such as oligo$(dT)_{20,}$ random hexamers), and reaction conditions of the SuperScript™III First-Strand System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.). The entire coding sequence (CDS) of the APP-cDNA (2313 bp) was PCR amplified from the single strand cDNA. The obtained purified DNA fragment corresponding to the CDS of the APP gene was subjected to the ligation reaction into the pcDNA™3.1/V5-His-TOPO®vector (Invitrogen, Carlsbad, CA, U.S.A.) and then introduced in One Shot®TOP10 chemical competent *Escherichia coli* strain for cloning by using the reagents and the transformation procedure of the TA Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The sequences of all primers used for PCR as well as for screening of insert are available upon request.

3. Results

3.1. Analysis of APP coding region

Isolation of mRNA from intact cultured fibroblasts followed by RT-PCR and cloning showed the presence of different isoforms of the entire coding sequence (CDS) of the APP-cDNA produced from a single APP gene by alternative pre-mRNA splicing and encode divers APP protein isoforms ranging from 120 to 770 amino acids with or without mutations and/or deletions in normal subject, control (sample # 1), in LNS (samples # 7,13), and in LNV (samples # 14,15) patients (Table 1).

Table 1
Isoforms of APP and mutations/deletions.

Samples[a]	Isoforms	Mutations and/or deletions
1	APP_{770}	No mutation
	APP_{770}	Mutation in exon 5: c.622T>C, p.V208A
	APP_{203}	Deletion starting after 102 bp of the 5′ end of exon 5 followed by a complete deletion of exons 6–16, and 104 bp of the 5′ end of exon 17. Mutation in exon 2: c.135A>G, p.N46D
	APP_{168}	Deletion starting after 93 bp of the 5′ end of exon 3 followed by a complete deletions of exons 4–16, and 59 bp of the 5′ end of exon 17. No mutation
7	APP_{770}	Mutations in exon 6: c.751G>A, p.G251D; exon 7: c.979A>G, p.N327S
	APP_{770}	Mutations in exon10: c.1249A>G, p.E417G; exon 11: c.1429T>C, p.I477T; exon 13: c.1657C>T, p.A553 V
	APP_{207}	Deletion starting after 49 bp of the 5′ end of exon 3 followed by a complete deletion of exons 4–15. Mutations in exon 1: c.21C>T, p.L8F; exon 3: c.268A>G, p.Q90R
	APP_{120}	Deletion starting after 27 bp of the 5′ end of exon 3 followed by a complete deletion of exons 4–16, and 138 bp of the end of exon 17. No mutation
13	APP_{770}	No mutation
	APP_{770}	Mutation in exon 12: c.1563delA, p.K522fs531X in exon 13
	APP_{751}	Mutation in exon 15: c.1930C>T, p.P644L
	APP_{751}	Mutations in exon 12: c.1557C>T, p.P520S; c.1570C>T, p.A524 V; exon 16: c.2062T>C, p.L688S
	APP_{216}	Deletion starting after 33 bp of the 5′ end of exon 3 followed by a complete deletion of exons 4–14, and 11 bp of the 5′ end of exon 15. No mutation
	APP_{168}	Deletion starting after 63 bp of the 5′ end of exon 3 followed by a complete deletion of exons 4–16 and 30 bp of the 5′ end of exon 17. No mutation
14	APP_{770}	No mutation
	APP_{770}	Mutation in exon 2: c.135A>G, p.N46D
	APP_{334}	Deletion starting after 9 bp of the 5′ end of exon 6 followed by a complete deletion of exons 7–15, and 15 bp of the 5′ end of exon 16. No mutation
	APP_{193}	Deletion starting after 42 bp of the 5′ end of exon 3 followed by a deletion of 209 bp of the 5′ end of exon 14. Complete deletion of exons 4–13. Deletion in exon 2: c.199delC, p.Q74fs86X in exon 3. Mutation in exon 3: c.242G>T, p.Q81H.
	APP_{175}	Deletion starting after 132 bp of the 5′ end of exon 2 followed by a complete deletion of exons 3–15, and 10 bp of the 5′ end of exon 16. Mutation in exon 18: c.2265 G>A, p.G756S
15	APP_{770}	No mutation
	APP_{770}	Mutations in exon 9: c.1215A>G, p.M406 V; exon 10: c.1380T>A, p.D427E; exon 16: c.2050A>G, p.H684R

[a] Samples used are the same ones as mentioned in Table 2: sample # 1 is normal subject, control; samples # 7,13 are LNS affected male patients; samples # 14,15 are LNV affected male patients.

3.2. Analysis of the genomic APP-DNA

In order to access the mutation of the genomic APP-DNA from normal subjects, controls (samples # 1–4), LNS (samples # 5–13), and LNV (samples # 14–16) patients, the sequencing analysis was performed of eighteen exons and flanking intronic sequences of human APP gene from the genomic DNA of intact cultured fibroblasts. No mutation was found.

3.3. Sequence analysis of the APP 5′ regulatory region

To screen for APP promoter variants, the −2693/+296 region (nucleotides 2355–5343) of the proximal (including exon 1) and distal APP promoter relative to the transcription start site (TSS), were amplified by PCR from genomic DNA of normal subjects, controls (samples # 1–4), LNS (samples # 5–13), and LNV (samples # 14–16) patients. The data obtained are summarized in Table 2.

4. Discussion

The human APP is a type I transmembrane glycoprotein with a long N-terminal extracellular region and a short C-terminal cytoplasmic tail [11,12]. The human APP gene is localized to chromosome 21 (21q21.2–3), spans approximately 240 kb and contains 18 exons. APP is the best known as the precursor molecule whose proteolysis generates beta amyloid (Aβ), a 39–42 amino acid peptide whose amyloid fibrillar form is the primary component of amyloid plaques found in the brains of individuals with Alzheimer's disease (AD) and Down's syndrome. APP consists of multiple structural and function domains: APP extracellular domain (exons 1–17: amino acid residues 1–699), APP transmembrane domain (exons 17–18: amino acid residues 700–723), and APP intracellular domain (exons 17–18: amino acid residues 724–770). Of interest, the Aβ sequence (exons 16–17: amino acid residues 672–713) is not conserved and is unique to APP (for details on the multiple structural and function domains of APP, see Refs. [11,12]. APP expression occurs ubiquitously, and the primary isoform varies according to cell and tissue type. Up to date, nine isoforms produced from a single APP gene by alternative pre-mRNA splicing and encoding proteins ranging from 365 to 770 amino acids have been reported [11,12]. The APP_{695} species is the most abundant isoform in neurons, while the APP-KPI isoforms (APP_{751} and APP_{770}) are predominantly expressed in glial cells, platelets, and peripheral tissue. Although APP has been the subject of much study since its identification, its physiological function remains largely undetermined. In the present study, I have identified for the first time in fibroblasts, several APP-mRNA isoforms encoding divers APP protein isoforms ranging from 120 to 770 amino acids from normal subject, control (samples # 1) as well as from LNS (samples # 7,13), and LNV (samples # 14,15) patients (Table 1). Interestingly, in addition to APP_{751} and APP_{770} (with or without mutations), several shorter APP isoforms (with or without mutations and/or deletions) ranging from 120 to 334 amino acids were also identified (Table 1). The mutations and/or deletions of multiple regions in several transcripts of APP could dramatically affect the stability and/or the function of the resulting APP proteins. Analysis by PCR amplification coupled with direct sequencing of eighteen exons and flanking intronic sequences of the CDS of APP gene from genomic DNA of these samples revealed no mutation. These findings suggest a role for epigenetic mechanisms and implications of the uncovered role of epigenetic components, such as DNA methylation, chromatin structure, histone modifications, etc. in the regulation of alternative pre-mRNA splicing [14,15]. The mRNA and protein isoforms produced by alternative processing of primary RNA transcripts may differ in structure, function, localization or other properties [14,15]. Indeed, it was reported that sequences of the upstream promoter of the human APP show differential patterns of methylation not only between different tissues, but also between different regions of the human brain [16]. My present results showed, for the first time, the real profile of APP isoforms in fibroblasts accounted for epigenetic mechanisms in the regulation of alternative APP pre-mRNA splicing. Recently, a role for epigenetic modifications in the regulation of alternative splicing has been reported [17]. The fact that divers APP isoforms with or without mutations and/or deletions have been found in fibroblasts of all subjects (normal control as well as LNS and LNV patients), it is therefore conceivable that changes in epigenetic regulation caused by genetic and environmental factors as well as life events and aging, could cause alterations in the regulation of alternative APP

Table 2
APP promoter mutation, HPRT mutation, HPRT deficiency, and Phenotype.

Samples[a]	APP promoter mutation[b]	HPRT mutation[c]	HPRT deficiency	Phenotype
1	−1023T>C Het	No	No	Normal
2	−1023T>C Het −1161A>G Het	No	No	Normal
3	−1023T>C Hom	No	No	Normal
4	−1023T>C Het	No	No	Normal
5	−1023T>C Hom	c.400G>A, p.E134 K	Complete	LNS
6	−1023T>C Het	c.532 + 1G>A, IVS7 + 1G>A	Complete	LNS
7	−1023T>C Het	Deletion in exons 2 and 3	Complete	LNS
8	−1023T>C Het	c.508C>T, p.R170X	Complete	LNS
9	−1023T>C Hom	c.151C>T, p.R51X	Complete	LNS
10	No	c.151C>T, p.R51X	Complete	LNS
11	−1023T>C Het	No mutation found in CDS	Complete	LNS
12	−1023T>C Het	No mutation found in CDS	Complete	LNS
13	−956A>G Hom −1023T>C Hom −1161A>G Hom −2224G>A Het −2335C>T Het	No mutation found in CDS	Complete	LNS
14	−1023T>C Het	c.500G>T, p.R167M	Partial	LNV
15	−1023T>C Het	c.500G>T, p.R167M	Partial	LNV
16	−1023T>C Het	c.500G>T, p.R167M	Partial	LNV

[a] Samples # 1–4 are normal subjects, controls; samples # 5–13 are LNS affected male patients; samples # 14–16 are LNV affected male patients.
[b] Numbering is relative to the transcription start site (TSS) with +1 as A of the nucleotide 5048 of the TSS in GenBank NG_007376. The nucleotides −956A, −1023T, −1161A, −2224G, and −2335C are the wild-type ones found in GenBank NG_007376. Het: heterozygous mutation, Hom: homozygous mutation.
[c] cDNA mutation numbering is based on GenBank NM_000194.2 with +1 as A of the ATG start codon.

pre-mRNA splicing and could result in an imbalance between different APP isoforms and some of these isoforms may be disease risk factors. As an example, mutations within and downstream from the alternatively spliced exon 10 of the microtubule-associated protein tau (MAPT) gene encoding the tau protein disrupt the 1:1 ratio required for mRNAs that include or exclude this exon. Exon 10 encodes the fourth of four microtubule-binding domains (R), and disruption of the balance between 4R-tau and 3R-tau isoforms results in hyperphosphorylation and aggregation of tau proteins into neurofibrillary tangles that are hallmarks of several neurodegenerative diseases such as AD [17]. My present results accounted for epigenetic mechanisms in the regulation of alternative APP pre-mRNA splicing may provide therefore a unique integrative framework for the pathologic diversity and complexity of AD, especially for late-onset AD. An accurate quantification of various APP isoforms in brain tissues for detection of initial pathological changes or pathology development is therefore needed. However, selective quantification of individual APP protein isoforms remains a challenge, because they simultaneously possess common and unique amino acid sequences. Recently, the use of APP quantification concatamer (APP-QconCAT) as an internal standard in multiple reaction monitoring (MRM) assay has been proven to be an effective method for quantification of APP isoforms [18].

In the current study, I also systematically analyzed the promoter sequences of APP. In principle, variations in promoter sequences can alter gene expression directly by altering a transcription factor-binding site or indirectly by changing the organization of chromatin. Promoter variants with effects on the transcriptional activity of certain human genes and in the regulation of alternative pre-mRNA splicing have been identified, and genetic association studies have suggested that some of these variants may be disease risk factors [19,20]. For screening of APP promoter variants, I focused on the region lying between −2693 and +296 (nucleotides 2355–5343) of the proximal (including exon 1) and distal APP promoter relative to the TSS since functional analysis and deletion mapping of the human and murine APP promoters have shown it to be sufficient for high level expression in various cell types [20]. This region was PCR amplified from genomic DNA of four normal subjects, controls (samples # 1–4), nine LNS affected male patients (samples # 5–13), and three LNV affected male patients (samples # 14–16). I have identified five genetic variants located in the distal APP promoter: −956A>G, −1023T>C, −1161A>G, −2224G>A, and −2335C>T (Table 2). Some of these variants such as −1023T>C, and −1161A>G was detected in both unaffected (samples # 1–4) and affected (samples # 5–9 and # 11–16) subjects while there were no polymorphisms in promoter sequences of one LNS affected male patient (sample # 10) (Table 2). These five independent polymorphisms in the APP promoter are novel and up to date, have not been reported. Since the number of patients and controls was small, I cannot yet exclude the existence of LNS-related variations in the APP promoter altering the transcriptional activity or alternative splicing. It is obvious that a systematic screening of the APP promoter sequences in extended LNS populations is necessary. However, obtaining a large number of LNS samples is problematic because it is an extraordinarily rare inherited neurogenetic disorder, with a prevalence of approximately three people in every million [21]. In anyway, these findings pointed out the unique differences between unaffected (samples # 1–4) and affected (samples # 5–16) subjects were the status of HPRT gene and/or the resulting HPRT enzyme activity (Table 2). Although there is no experimental evidence at present proven the direct link between LNS and APP, however, based on the results summarized in Tables 1 and 2, it is conceivable that in LNS, epigenetic modifications, due to gene-gene interactions (epistasis) [22] between mutated HPRT and APP genes, could affect the regulation of alternative APP pre-mRNA splicing in favor of APP isoforms responsible for the disease. The severity of the affection would depend on how mutations ultimately alter interactions between mutated HPRT and APP genes. The type of mutation and its location in HPRT gene is therefore an important factor for provoking disease (LNS or LNVs) not only through its effect on residual HPRT enzyme activity but also through its effect on interactions between mutated HPRT and APP genes. For the same type of mutation in HPRT gene, response to epigenetic modifications due to epistasis may be different from one patient to another and this could explain for the manifestation of different clinical phenotypes from different patients and also from different affected family members [4,23,24]. Here, the quantification of APP isoforms in brain tissues by means of APP-QconCAT method [18] may be useful for identification of the ones responsible for the disease and prove therefore the direct link between LNS and APP. It is important to note that epistasis is important, ubiquitous, and has become a hot topic in complex disease genetics in recent years such as AD, schizophrenia, autism, type 2 diabetes, sporadic breast cancer, etc. and even common for determining phenotypes for a number of rare Mendelian diseases such as cystic fibrosis, sickle-cell anemia, Hirschsprung disease, etc. [25]. However, the data supporting epistasis in complex human diseases are emerging slowly. This is due to different difficulties that we face in detecting and characterizing epistasis such as challenges of modeling non-linear interactions, and in interpretation of results [22,25].

Overall, although there is no experimental evidence at present proven the direct link between LNS and APP but my results showed, for the first time in fibroblasts from normal subjects as well as from LNS and LNV patients: (a) the real profile of APP isoforms accounted for epigenetic mechanisms in the regulation of alternative APP pre-mRNA splicing and (b) five novel independent polymorphisms in the APP promoter. A role for epistasis between mutated HPRT and APP genes affecting the regulation of alternative APP pre-mRNA splicing in LNS is suggested. Through a massive research effort over the last two decades, it has now become clear that APP and its fragments play diverse roles in development and cell growth, intercellular communication, signal transduction, nuclear signaling, and structural and functional plasticity. The importance of APP as a molecular hub at the center of interacting pathways and it is not surprising therefore that altered APP processing may affect brain function through a host of altered cellular and molecular events. My findings may provide new directions not only for investigating the role of APP in neuropathology associated with HPRT-deficiency in LNS but also for the research in neurodevelopmental and neurodegenerative disorders by which various APP isoforms involved in the pathogenesis of the diseases such as AD.

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