The Human Folate Receptor 1 Gene: Molecular Diagnostic of Folate Deficiency

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Article ID: WMC003461
Article Type: Research articles
Submitted on: 08-Jun-2012, 02:31:43 AM GMT   Published on: 09-Jun-2012, 04:06:13 PM GMT
Article URL: http://www.webmedcentral.com/article_view/3461
Subject Categories: MEDICAL EDUCATION
Keywords: Folate deficiency, FOLR1 gene, Coding sequence, PCR, Sequencing, Cloning
How to cite the article: Nguyen KV, Nguyen R, Nyhan WL. The Human Folate Receptor 1 Gene: Molecular Diagnostic of Folate Deficiency. WebmedCentral: International Journal of Medicine and Molecular Medicine 2012;3(6):WMC003461
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Source(s) of Funding:
University of California, San Diego
Competing Interests:
No

International Journal of Medicine and Molecular Medicine is an associate journal of Webmedcentral.
The Human Folate Receptor 1 Gene: Molecular Diagnostic of Folate Deficiency

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Abstract

Folate is a water-soluble vitamin of the B complex group, and is required for optimal health, growth, and development. In human, it cannot be synthesized de novo, hence, dietary sources must meet metabolic needs. Folate deficiency is one of the major dietary health problems worldwide and is associated with a variety of diseases including anaemia, birth defects, cardiovascular disease, and neurological conditions. In the present study, we report an application of the human folate receptor 1 gene (FOLR1) for molecular diagnostic of folate deficiency. The cloning of the entire coding sequence (CDS) of the FOLR1 gene is also reported.

Introduction

Folate and folic acid are forms of a water-soluble B vitamin. Folate occurs naturally in food, and folic acid is the synthetic form of this vitamin [1,2]. Folate is a B vitamin essential for brain metabolism. It is involved in essential one-carbone methyl transfer reactions and crucial for a variety of biological processes, such as synthesis and repair of DNA [3,4], regulation of gene expression [5], and interaction of amino acids and neurotransmitters [6,7]. Folate deficiency is one of the major dietary heath problems worldwide and is associated with a variety of diseases including anaemia, birth defects, cardiovascular disease, and neurological conditions [8-10]. Humans require 400-600 µg of dietary folate per day, and children and adolescents are the population at the greatest risk of suffering nutritional folate deficiency. Dietary folate is taken up in the gut, metabolized in the liver to 5-methyl tetrahydrofolate (MTHF), then distributed by the blood stream. Cellular uptake of MTHF is mediated by the proton-coupled folate transporter (PCFT; SLC 46A1 [MIM 611672]), the reduced folate carrier (RFC; SLC 19A1 [MIM 600424]), and by two glycosylphosphatidylinositol (GPI)-anchored receptors, folate receptor alpha (FRα [MIM 136430]) and beta (FRβ [MIM 136425]) [11]. FRα is distributed mainly at epithelial cells, such as choroid plexus, lung, thyroid, and renal tubular cells, whereas FRβ is located mainly within mesenchymal derived cells, such as blood cells. Both are high-affinity receptors that function at the physiological nanomolar range of extracellular folate concentrations. FRα is encoded by the folate receptor 1 gene (FOLR1). This gene product is a secreted protein that either anchors to membranes via a GPI linkage or exists in a soluble form. The FOLR1 gene is located on the long (q) arm of chromosome 11 between positions 13.3 and 14.1: 11q13.3-q14.1. This gene is composed of seven exons that span 6.8Kb; exons 1 through 4 encode the 5’ untranslated region (UTR) and exons 4 through 7 encode the open reading frame and the 3’UTR. Due to the presence of two tissue specific promoters P1 and P4 up-stream from exon 1 and exon 4 respectively, multiple transcription start sites, and alternative splicing of exons, several transcript variants are derived from this gene. These variants differ in the lengths of 5’ and 3’ UTR, but they encode an identical amino acid sequence [12,13]. In this study, an application of the FOLR1 gene for molecular diagnostic of folate deficiency is reported.

Materials and Methods

Isolation of genomic DNA and amplification: The RNA-free genomic DNA samples were isolated from whole peripheral blood using the Puregene® DNA Purification Kit (Gentra System, Minneapolis, Minnesota, U.S.A.). The DNA concentration was determined by using the ND-1000 spectrophotometer NanoDrop® device. For genomic characterization, by using the polymerase chain reaction (PCR) technique [14,15], each of the four exons and flanking intronic sequences of the coding sequence of the human FOLR1 gene (GenBank NG_015863) were first PCR-amplified by means of primers designed to be specific to the intronic genomic sequences. PCR primers employed and amplicon sizes are shown in Table 1. Amplification was conducted using a DNA thermal cycler (Verit 96 well Thermal Cycler, Applied Biosystems Life Technologies Corporation, Carlsbad, CA, U.S.A.). The reaction was conducted in a total volume of 50µL with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). The reaction was conducted in a total volume of 50µL with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). The reaction was conducted in a total volume of 50µL with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.).
analyzed by electrophoresis on a 1.5% agarose gel. The PCR product was then isolated and purified by using the reagents and conditions according to the manufacturer’s instructions of the QIA quick® PCR Purification Kit (QIAGEN Sciences, Maryland, U.S.A.). The obtained purified PCR product was sequenced using the same primers as for PCR and the ABI DNA sequencer (Applied Biosystems).

**Isolation of mRNA and Amplification:** The mRNA was isolated from intact cultured fibroblasts using the FastTrack® 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA, U.S.A.). The mRNA concentration was determined by using the ND-1000 spectrophotometer NanoDrop device. A first strand 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 RT gene-specific primer used for cDNA synthesis of the FOLR1 gene is shown in Table 1. The entire coding sequence (CDS) of the FOLR1-cDNA was amplified from the single strand cDNA using specific primers as shown in Table 1. The reaction was conducted in a total volume of 50µL with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). Amplification conditions were as follow: Denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and elongation at 72°C for 1 min, each for 35 cycles. Amplification was conducted using a DNA thermal cycler (Veriti 96 well Thermal Cycler, Applied Biosystems Life Technologies Corporation, Carlsbad, CA, U.S.A.). The PCR product was analyzed by electrophoresis on a 1.5% agarose gel. The 774bp DNA fragment of the CDS of the FOLR1 gene was obtained. The PCR product was then isolated, and purified by using the reagents and conditions according to the manufacturer’s instructions of the QIA quick® PCR Purification Kit (QIAGEN Sciences, Maryland, U.S.A.). The obtained purified PCR product was sequenced using the same primers as for PCR and the ABI DNA sequencer (Applied Biosystems Life Technologies Corporation, Carlsbad, CA, U.S.A.).

**Cloning:** The obtained purified DNA fragment corresponding to the CDS of the FOLR1 gene (774bp) was subjected to the ligation reaction into the pCR®II plasmid vector of the TA Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The reagents of this kit and the reaction conditions used are according to the manufacturer’s recommendations. The ligation product was then introduced in One Shot®TOP10 chemically competent E.coli strain by using the reagents and the transformation procedure of the TA Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The screening for insert was performed using blue-white selection. The sequencing of insert was performed using the ABI DNA sequencer (Applied Biosystems Life Technologies Corporation, Carlsbad, CA, U.S.A.). The resulting vector was termed (1) (pCR®II/ FOLR1-CDS).

**Results and Discussion**

**Analysis of the genomic FOLR1 DNA:** For molecular diagnostic purpose, the PCR technique coupled with direct sequencing was applied. The sequencing analysis of the four exons and flanking intronic sequences of the coding region of the human FOLR1 gene from the DNA genomic of whole peripheral blood from normal subject was performed. The results of the present study show that all PCR products were successively amplified (Figure 1A).

**Amplification and Cloning of the FOLR1 coding sequence:** Isolation of mRNA from culture fibroblasts from normal subject followed by RT and amplification via PCR showed a 774bp fragment of the CDS of the FOLR1-cDNA (normal size) (Figure 1B). The sequencing results of the FOLR1-CDS insert from the vector (1) (pCR®II/ FOLR1-CDS) showed a correct sequence (data not shown). The cloning of the CDS of the FOLR1 gene into the pCR®II plasmid vector was successful.

**Application for Molecular Diagnostic of Folate Deficiency:** Mammals cannot synthesize folates; hence, dietary sources must meet metabolic needs. Folate deficiency is one of the major dietary health problems worldwide, and is prevalent in underdeveloped countries [8], and even in the Western world, subtle deficiency is a public health problem that is most notable in its association with neural tube defects in the developing embryo [16,17]. In addition, FRα is of central importance for folate transport across the blood-brain barrier via the choroid plexus. Autoantibodies against folate receptors were identified as the cause of the infantile-onset cerebral folate deficiency (CFD) syndrome [18]. Recently, mutations in the FOLR1 gene, encoding the FRα, can cause brain-specific folate transport deficiency leading to a treatable (by folic acid supplementation) neurodegenerative disorder associated with disturbed myelin metabolism in early childhood were described [19-21]. Indeed, by
detecting a decrease of the MTHF levels in cerebrospinal fluid (CSF) but normal plasma MTHF levels from patients, these investigators have identified four mutations in FOLR1 gene from the DNA genomic of whole peripheral blood from patients (Table 2). However, the GenBank Accession No. for the genomic mutation numbering was not mentioned from these articles [19-21] (see (a) in Table 2), and the PCR conditions as well as the primer sequences used for mutation analysis of FOLR1 gene were not fully mentioned [20] or were available upon request only [19,21]. Moreover, the GenBank NM_016730 reference sequence (see (a) in Table 2) used by these authors [19-21] for the cDNA mutation numbering was, according to the NCBI notice, permanently suppressed because currently there is insufficient support for the transcript. Taking into account these considerations, in the present study, the PCR conditions, primer sequences, and the synthesis of the CDS of the FOLR1 gene as well as its cloning into the pCR®II plasmid vector, were reported. In addition, by referring to the GenBank NG_015863 and NM_00082, the location of the mutations in FOLR1 gene identified by these authors [19-21] were updated and shown in Table 2 (see (b) in Table 2). The resulting vector (1) (pCR®II/ FOLR1-CDS) obtained from the cloning of the CDS of the FOLR1 gene into the pCR®II plasmid vector is useful for diver applications. It can be used (a) to perform easily amplifications by PCR of any exon of the FOLR1 gene for diver purposes such as for use as a component of any construct, for the construction of labeled nucleotide probes, for FOLR1 mRNA expression quantification by means of RT-PCR or real-time RT-PCR; (b) to construct the standard curve for FOLR1 mRNA expression quantification by means of RT-PCR or real-time RT-PCR techniques; (c) to perform the site-directed mutagenesis for characterizing the dynamic, complex relationships between protein structure and function, for studying gene expression elements, and for carrying out vector modification.

All folate-related biological reactions occur within the context of one-carbon-donor (methyl, methylene, and formyl groups), methyl transferase-catalyzed reactions. As a cofactor or coenzyme, folate plays key biological roles in a variety of physiologic processes. These include: (a) Maintenance and repair of the genome. As a coenzyme, folate acts as an acceptor and donor of one carbon unit in reactions critical in the metabolism of nucleic acids. Folate has an essential role within the biosynthetic pathways of both purines and pyrimydines. (b) Regulation of gene expression. Folate influences the regulation of gene expression, by participating in the synthesis of S-adenosylmethionine (a methyl-group donor involved in the methyllation of a number of sites within DNA and RNA). Folate is involved in the actions of methyl cytosine-phosphate diester-guanine-binding protein 2 (MECP2), which influences chromatin structure and remodeling and gene silencing, by binding to methylated DNA. If the DNA is not methylated, the MECP2-mediated gene “silencing” does not occur, and the integrity of the genome is disrupted; (c) Amino-acid metabolism. Folate serves in the generation of glycine, glutamic acid, and methionine, and plays an essential role in the regulation of homocysteine serum levels; (d) Myelin. Through its role in S-adenosylmethionine synthesis, folate participates in phospholipids biosynthesis including the formation of myelin; (e) Neurotransmitter synthesis. Folate is important for the biosynthesis of serotonin, catecholamines, and melatonin. The striking evidence about the essential roles of folate in the maintenance of human health, growth, and development led to the ruling by the Food and Drug Administration that all enriched grain products, such as flour, bread, and corn meal, should be fortified with synthetic folic acid. This ruling was implemented as of January 1998 in the United States [22]. Diverse clinical conditions are associated with abnormal body folate status, paralleling the diversity of folate’s biological roles. They range from genetic syndromes defined prior to conception (e.g., Down syndrome), to malformations that develop during embryogenesis (e.g., neural tube defects), to disorders that are acquired postnatally and may be progressive (e.g., cerebral folate deficiency). With the exception of congenital malformation, neurologic presentations are not specific. However, because the majority of patients respond to treatment with folic acid, pediatric neurologists should remain vigilant to the possibility of deficiencies of folate, and consider it before drawing any conclusions about the cryptogenic etiologies of unexplained cases. Therefore, the development of a molecular diagnostic for folate deficiency by identification of mutations in FOLR1 gene from the DNA genomic of whole peripheral blood from patients as described in the present study is important to start early folic acid supplementation to prevent worsening of their clinical condition.
Conclusion

Folate plays a key role in nucleic-acid synthesis, in maintenance and repair of the genome, and in amino-acid and neurotransmitter metabolism. Clinical phenotypes related to folate deficiency are diverse, which reflects the variety of the underlying pathophysiologic mechanisms, the complex metabolic and absorption pathways, and the gene-gene and gene-environment interactions. Clinical consequences can develop as a result of suboptimal nutritional conditions, decreased availability or enzymatic utilization, or impaired DNA elaboration and gene expression, or within the interactions of these mechanisms. Therefore, this study supports the view that screening for plasma as well as CSF MTHF levels followed by a molecular diagnostic for folate deficiency via identification of mutations in FOLR1 gene from the DNA genomic of whole peripheral blood from patients is important to start early folic acid supplementation to prevent worsening of their clinical condition. Currently, a lack of commercially available testing for the determination of cerebrospinal fluid folate status is the major obstacle delaying further progress in these directions.

References

Illustrations

Illustration 1

Fig. 1A: PCR amplification of the four exons 1-4 and flanking intronic sequences of the coding sequence of the human FOLR1 gene. M: Marker (kb); Lane 1: Fragment 1 (exon 1: 319bp); Lane 2: Fragment 2 (exon 2: 321bp); Lane 3: Fragment 3 (exons 3 and 4: 725bp).

Illustration 2

Fig. 1B: PCR amplification of the entire coding sequence (CDS) of the human FOLR1 gene. M: Marker (kb); Lane 1: Fragment 1 (CDS: 774bp).
Illustration 3

Table 1

Exon-flanking oligonucleotide primer sequences used for the amplification of all four exons 1 to 4 of the human FOLR1 gene and the primers sequences used for the FOLR1-CDS synthesis.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Nucleotide sequence (5’®3’)</th>
<th>Location (a)</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (forward)</td>
<td>CCCTGCACACAACTTAAGG</td>
<td>7542-7560</td>
<td>Exon 1 (319bp)</td>
</tr>
<tr>
<td>2 (reverse)</td>
<td>GGGTTTGGCATTCCATGTC</td>
<td>7841-7860</td>
<td></td>
</tr>
<tr>
<td>3 (forward)</td>
<td>GTGGAATATTCTGGCTGTGC</td>
<td>10648-10667</td>
<td>Exon 2 (321bp)</td>
</tr>
<tr>
<td>4 (reverse)</td>
<td>CTGCAGAGATGCTGGAAAG</td>
<td>10949-10968</td>
<td></td>
</tr>
<tr>
<td>5 (forward)</td>
<td>CAGCTCCAGTTCTATTCCGG</td>
<td>10979-10997</td>
<td>Exons 3 and 4 (725bp)</td>
</tr>
<tr>
<td>6 (reverse)</td>
<td>GACCATGGAGCAGGAACC</td>
<td>11686-11703</td>
<td></td>
</tr>
<tr>
<td>7 (RT)</td>
<td>CTGAACAGGGCAG</td>
<td>11652-11664</td>
<td></td>
</tr>
<tr>
<td>8 (forward)</td>
<td>ATGGCTCAGCGGATGACAAC</td>
<td>26-45</td>
<td>CDS (774bp)</td>
</tr>
<tr>
<td>9 (reverse)</td>
<td>TCAGCTGAGCAGCCACAGC</td>
<td>781-799</td>
<td></td>
</tr>
</tbody>
</table>

(a) Primer sequences numbering is based on GenBank NG_015863. The primers Nos. 1 to 6 are used for the synthesis of the four exons 1 to 4 and flanking intronic sequences of the coding sequence of the human FOLR1 gene. The primers Nos. 7 to 9 are used for the synthesis of the entire coding sequence (CDS) of the FOLR1- cDNA.
Illustration 4

Table 2

Identification of mutations in FOLR1 gene responsible for cerebral folate transport deficiency.

<table>
<thead>
<tr>
<th>Mutation No.</th>
<th>Mutation (a)</th>
<th>Mutation (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>exon 2: g.130-147dup, c.130-147dup,</td>
<td>exon 1: g.7746-7763dup,</td>
</tr>
<tr>
<td></td>
<td>p.K44-P49dup</td>
<td>c.155-172dup, p.K44-P49dup</td>
</tr>
<tr>
<td>2</td>
<td>exon 3: g.3242T&gt;C, c.313T&gt;C,</td>
<td>exon 2: g.10858T&gt;C, c.338T&gt;C,</td>
</tr>
<tr>
<td></td>
<td>p.105C&gt;R</td>
<td>p.105C&gt;R</td>
</tr>
<tr>
<td>3</td>
<td>exon 3: g.3281C&gt;T, c.352C&gt;T,</td>
<td>exon 2: g.10897C&gt;T, c.377C&gt;T,</td>
</tr>
<tr>
<td></td>
<td>p.118Q&gt;X</td>
<td>p.118Q&gt;X</td>
</tr>
<tr>
<td>4</td>
<td>exon 5: g.3755C&gt;A, c.525C&gt;A,</td>
<td>exon 4: g.11371C&gt;A, c.550C&gt;A,</td>
</tr>
<tr>
<td></td>
<td>p.175C&gt;X</td>
<td>p.175C&gt;X</td>
</tr>
</tbody>
</table>

(a) The cDNA mutation numbering is based on GenBank NM_016730 with +1 as A of the ATG start codon. The mutations Nos. 1 and 2 were respectively identified by Steinfeld et al. [20] and Duenas et al. 2011 [21]. The mutations Nos. 3 and 4 were respectively identified by Cario et al. 2009 [19] and Steinfeld et al. 2009 [20].

(b) Genomic mutation numbering is based on GenBank NG_015863. The cDNA mutation numbering is based on GenBank NM_000802 with +1 as A of the ATG start codon.
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