Focal glial detection coincides with precedes amyloid plague formation in APPPS1 transgenic mice by PCR

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Abstract

The identification and sequencing of Amyloid Precursor Protien (APP) and presenilin (PS) opened the door for the engineering of transgenic mouse models to study pathogenic mechanisms of Alzheimer Disease (AD). The first successful mouse models over-expressed human APP with an Familiar AD (FAD) linked mutation in the brain. These mice exhibit Aß plaques, neuron loss, dystrophic neurites, inflammatory responses, learning impairments and deficits in synaptic transmission and/or long-term potentiation. The genotypes of all offspring of APP/PS1 mutant mice are analysed by Polymerise Chain Reactions. Generally there are two possibilities to analyse the DNA. The First, primers for APP or PS1 was used separately assuming that both genes are integrated into the transgene. The second possibility is to do both amplifications in one PCR. Transgene mapping revealed that both transgenes were integrated at the lower arm of chromosome 2 between 40 and 60 cM. The result showed a clear band of APP gene. By using many gel concentrations(1%, 2%, 5%). The 2% gel concentration is the best to visualize the band in 150 V at 1 hour, in order to optimize the method. The Polymerise Chain reaction method (PCR) also had been optimized, in order to have a band of APP. Considering the number of studies that rely on the detection of AD pathology, it is surprising to find such high variability in the APPPS1gene PCR of key AD-related markers across pretreatments in adjacent Aβ Accumulations of gene manipulation.

Keyword: Amyloid precursor protein , Polymerise chain reaction Technique.

التشخيص المتزامن لبقع الاملويد المتكونة في الخلايا العصبية المركزية للفئران المعدلة وراثيا من نوع APPPS1 باستعمال تقنية PCR

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الخلاصة

أن عملية تشخيص التسلسل الجيني لبروتينات بادئ الاملويد APP والبرسنيلن PS1 فتحت المجال للهندسة الوراثية لعمل فئران معدلة وراثيا تظهر أعراض مرض الزهايمر، مثل بقع تجمع البروتين الاميلويد بيتا Aβ ، وضرر الخلايا العصبية ، والالتهابات ، وفشل إلية الذاكرة المقاس بالتحفيز طويل الأمد LTP. تم دراسة تحليل النمط الجيني لكل الفئران المطفرة جينيا لبروتين بادئ الاملويد باستخدام تقنية تفاعلات التسلسل الحراري المضاعف لل DNA (PCR)، باستعمال برايمرات Primers مصممة لتضاعف هذا البروتين فقط . أظهرت النتائج التميز الواضح لحزمة ال DNA بروتين بادئ الاملويد في الفئران المعدلة وراثيا مقارنتا بفئران السيطرة؛

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وكذلك للأفراد الأخرى ضمن المستعمرة التي لم ترث هذا الجين ضمن قوانين مندل للوراثة ؛ على هلام الترحيل الكهربائي بتركيز 2% بقدرة 150V لمدة ساعة. وبما أن مثل هذه الدراسات التي توضح ميكانيكيا ت حدوث الزهايمر وعلاقته ببروتين بادئ الاملويد، كان من الواجب تحسين طريقة تحليل النمط الجيني لبروتين APP ، كمفتاح لتمييز الفئران التي تحمل الجين من غيرها، وبالتالي تظهر تجمع نواتج تكسر البروتين وهي Aβ نتيجة التغييرات الجينية .

Introduction

Amyloid precursor protien is a type-I integral transmembrane glycoprotein that is ubiquitously expressed, but the physiological function of APP in the brain remains unclear. It has been proposed to have functions in transmembrane signal transduction, calcium regulation, cell proliferation, cell adhesion, neurite outgrowth and synaptogenesis. This is reflected in APP knock out mice, which show cognitive deficits and reduced synaptic plasticity [1,2]. APP is processed by α-, β- and γ-secretase. It is known that, non amyloidogenic cleavage pathway, α - secretase cleaves within the Aβ region to produce the soluble α -APP (sAPP) and the 83 amino acid COOH-terminal fragment C83 [3,4]. Alternatively, processing with ß-secretase produces a 99 amino acid C-terminal (C99), which includes Aß [5] and a longer form of sAPP. Both the C83 and C99 fragments remain anchored in the membrane where they are processed by γ -secretase to produce p3 from C83 or Aβ from C99. β-Secretase cleavage occurs within the membrane, but is not sequence specific resulting in Aß peptides of varying length. The most common form is Aß40, but there is always a percentage of Aß42, which is less soluble and more likely to form toxic aggregates [6]. Extensive research has revealed that γ- secretase is a multi-protein high molecular weight complex and consists of presenilin-1 (PS1), nicastrin (Nct), anterior pharynx-defective phenotype (APH-2) and PSenhancer (PEN-2) all of which are essential for its function [6-10].

Missense mutations on the APP gene that have been linked to Familiar Alzheimer Disease (FAD) are all located at the cleavage sites of α-, β- and γ-secretase. The different mutations have been named after the geographic area the FAD inheriting family lived in, e.g. Swedish, Dutch or London [11-13]. FAD mutations at the α- or β- secretase cleavage site favor ß-secretase cleavage, and therefore increase Aß production over p3 production . Mutations at the γ-site promote the production of Aβ42 over Aß40 [14]. Both increased Aß production and a higher rate of Aß42 are responsible for increased deposition of Aß plaques.

The identification and sequencing of APP and presenilin opened the door for the engineering of transgenic mouse models to study pathogenic mechanisms of AD. The first successful mouse models over-expressed human APP with an FAD linked mutation in the brain. These mice exhibit Aß plaques, neuron loss, dystrophic neurites, inflammatory responses, learning impairments and deficits in synaptic transmission and/or long-term potentiation [4-9] .

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by progressive memory loss and decline of cognitive functions. Histopathological hallmarks include extracellular amyloid peptide (Aβ) deposition in neuritic plaques, and intracellular deposits of hyperphosphorylated Tau, causing formation of neurofibrillary tangles and finally neuronal death. Aβ peptides are generated from amyloid precursor protein (APP) by sequential actions of two proteolytic enzymes, i.e. the β- site APP cleavage enzyme (BACE1) and the γ-secretase [15,16]. Their formation and eventual deposition represents a key feature and possibly the triggering mechanism of AD. The importance of Aβ formation was instigated by dominantly inherited familial forms of AD that are linked to APP mutations in or close to the β- and γ-secretase cleavage sites [17]. This made it possible to generate transgenic mouse models of cerebral amyloidosis and AD-like histopathology, i.e. amyloid plaques and cerebral amyloid angiopathy (CAA) [18-20].The severity and onset time of AD like pathology is influenced by the level of transgene expression and the specific mutation. Other models combine the insertion of mutated APP with an insertion of mutated PS1, or insert APP with two mutation sites to have an even stronger phenotype [21,22]. None of these Aß depositing mouse models develop tau filaments. This has only been shown in mice expressing mutant human tau protein or by combining Aß deposition with a mutation in the tau gene [23-25].

Materials and Methods

Genotyping of APP/PS1 Transgenic Mice: The transgenic mice get from the Hariline Institute in Northern Ireland. The genotypes of all offspring of APP/PS1 mutant mice are analysed by PCR. Generally there are two possibilities to analyse the DNA. First it can be use primers for APP or PS1 separately assuming that both genes are integrated into the transgene. The second possibility is to do both amplifications in one PCR. In our lab, specific primers were performed for PCR(table 1). Only if it is required it can make another PCR with PS1 specific primers. In addition, another protocol for PCR with APP- and PS1-primers together was done [11,26,27,28,29,30].

Table 1- APPPS1 Primers. Prepared by using Primer3 program [www.primer3.co.us/cal

Name	Forward/reverse	Gene	Sequence 5′ →3′
PS1F	F	HuPsen1	CAG GTG CTA TAA GGT CAT CC
PS1R	R	HuPsen1	ATC ACA GCC AAG ATG AGC CA
APPfor	F	APP	GAA TTC CGA CAT GAC TCA GG
APPrev	R	APP	GTT CTG CTG CAT CTT GGA CA
APP1	F	APP	CGA CAG TGA TCG TCA TCA CCT
APP4	R	APP	CTT AGG CAA GAG AAG CAG CTG

Isolation of genomic DNA from tissue samples: Approximately 1 cm of the mice tail was mixed with 180 µl ATL lysis buffer (Qiagen) and 18 µl Proteinase K (Qiagen). Incubated at 56°C over night in a Thermomixer at 650 rpm. Then, Diluted 1:200 with ddH_2O to a total volume of 200 µl .

PCR Procedures:

1. APP-PCR with the *Taq* PCR Master Mix Kit (Qiagen)

the followings components were mixed on ice:

12.5 µl *Taq* PCR MasterMix per sample, plus 10% at the end

10.5 µl ddH2O

0.1 µl Primer APPfor (100 pmol)

0.1 µl Primer APPrev (100 pmol)

Add 1.5 µl diluted DNA sample

For positive control, sample of known APP tg mouse was added. For negative control, distilled water was added.

APP-PCR program

1.	95°C	4 min	As denaturizing step
2.	95°C	45 sec	
3.	58°C	45 sec	
4.	72°C	45 sec	2-4 steps as elongating steps
5.	72°C	5 min	as extension
6.	4°C	forever	as termination step

Steps (2- 4) 34 cycles was repeated [8]

2. PS1-PCR with the *Taq* PCR Master Mix Kit (Qiagen)

the following components were mixed on ice:

12.5 µl *Taq* PCR MasterMix

10.5 µl ddH2O

0.1 µl Primer PS1F (100 pmol)

0.1 µl Primer PS1R (100 pmol)

1.5 µl DNA was diluted.

PS1-PCR program

1.	95°C	4 min	As denaturizing step
2.	94°C	45 sec	
3.	58°C	45 sec	

4. 72°C 45 sec 2-4 steps as elongating steps
5. 72°C 10 min as extension step
6. 4°C forever terminal step

Steps (1- 3) 35 cycles was repeated [5,28]

3. Alternatively: Double Identification of APP and PS1

the following components were mixed on ice:

10 µl H2O
2 µl $MgCl_2$ (20 mM)
2 µl 10 x PCR Buffer
2 µl 10 mM dNTPs (2 mM each)
1 µl Primer PS1F (20 µM)
1 µl Primer PS1R (20 µM)
1 µl Primer APP1 (20 µM)
1 µl Primer APP4 (20 µM)
0.2 µl *Taq* polymerase (Roche, 5U/µl)

An appropriate amount (2µl) of DNA was added.

APP/PS1-PCR program

1. 95°C 4 min as denaturizing step
2. 95°C 40 sec
3. 58°C 1 min
4. 72°C 1 min 2-4 as elongating steps
5. 72°C 5 min as extension step
6. 4°C forever as terminal step

Steps (2 -4). 30 cycles was repeated [5,26]

Gel casting and electrophoresis

Five µl of 6 x loading dye was added to 25 µl of PCR product, and analysed samples on a 1.5 % -TBE agarose gel (approx. 150 V; 1 h). Agarose gel (2%) was prepared by adding 1.6g agarose in 80ml, and 50% TBE buffer. Horizon 11-14 electrophoresis system was used. Gel thickness 5mm was appropriate. Comb was chosen depending on sample size (25µl volume of sample appropriate to 10 teeth comb). Black blocks inserted in electrophoresis chamber; and then the combs; buffer is available in tank, dilute 50:50. Agarose was added, then boiling by the microwave until clear liquid after cooling processes at the room temperature, then, 6µl Ethidium Bromide was added. the gel was poured, had waiting 40 min to cool, comb had been taken out, covering the gel with 1 liter of TBE, waiting 20 min, and Loading dye 10% was added to sample ,vortex briefly to mix samples plus positive control, negative control (master mix with water), and plus pocket for 'ladder' were preserved at -20° c.

Loading gel

Ladder was loaded 5-10µl depending on comb size. Sample 20-35µl per well was loaded by using micropipette in appropriate tips. Power supply had switch on (BioRad) at Voltage (150 V) for 60 min, and then the gel carefully was taken out (in container) to gel reading room in a UV light scanner by used a specific software to record.

Results

Several transgenic lines were generated. The transgene segregation followed the expected mendelian inheritance. Transgene mapping revealed that both transgenes were integrated at the lower arm of chromosome 2 between 40 and 60 cm. The result showed a clear band of APP gene (Figure 1.) After using many gel concentrations(1%, 2%, 5%). 2% gel concentration was the best to visualize the band in 150 V at 1 hour, in order to optimize the method. The Polymerase Chain Reaction method PCR also had been optimized, in order to have a band of APP. By following the steps of Qiagen kit, choosing the concentrations of addings become possible. The primer type and quality had been chosen by using the Primer 3 program software (California genetic institute software)

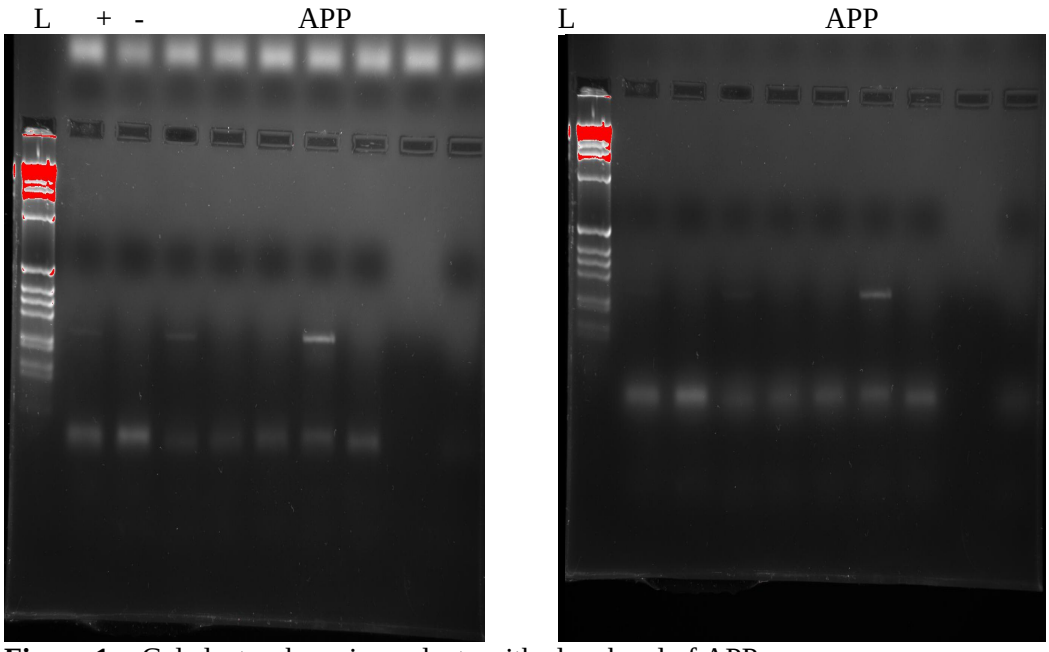

Figure 1-. Gel electrophoresis products with clear band of APP gene.
L: ladder, (+) positive control, (-) negative control, (APP) Amyloid precursor protein.
Gel electrophoreses concentration (2%). The voltage was chosen 150 V for 60 min.

Discussion

Considering the number of studies that relies on the detection of AD pathology, it is surprising to find such high variability in the APPPS1gene PCR of key AD-related markers across pretreatments in adjacent Aβ Accumulations of gene manipulation. Whereas many groups have compared the sensitivity of different PCR technique in many transgenic animals, as it is known [1-10], this study is the first to quantify the APP transgenic mice.

APPPS1 mice are model of cerebral amyloidosis, and not directly of AD, as the mice do not model the tau pathology and robust neurodegeneration observed in AD. Neuron loss in the APPPS1 model seems to be similar to previous APP and APPPS1 transgenic mouse models, in which neuron death occurs only in close proximity to congophilic amyloid, giving rise to no significant global neuron loss in the neocortex and modest and mouse-model-specific neuronal loss in the hippocampal subregions [31-34].

Several APP and APPPS transgenic mice have been described in the previous studies. The present APPPS1 mouse combines several advantages of previous transgenic lines. (i) Mice have been generated on a pure C57BL/6J background that is known to reduce the variability of Aβ metabolism and deposition [35]. More recently, the mouse model has also been expanded into a BALB/cJ background. (ii) At least in young mice, no gender effects in Aβ level and amyloid deposition have been noted. (iii) Several APPPS1 lines have been generated that help to control for unwanted transgene insertion site effects. (iv) APPPS1 mice breeded well, similar to wild-type C57BL/6J mice. (v) The APP and PS1 transgenes are coexpressed and the integration site has been located. (vi) The early onset of amyloid pathology allows a rapid readout and facilitates testing of therapeutic amyloid-targeting strategies. (vii) APPPS1 mice are a model of parenchymal amyloidosis and a welcome addition to the recently generated APP Dutch mouse model that develops only vascular amyloid [36]. Because of these reasons, we anticipate that this new APPPS1 mouse model, now distributed to more than 30 laboratories worldwide, will be broadly valuable for addressing the various issues about the role of cerebral amyloidosis in AD and other proteopathies. Considering the number of studies that rely on the detection of AD pathology, it is surprising to find such high variability in the APPPS1gene PCR of key AD-related markers across pretreatments in adjacent Aβ Accumulations of gene manipulation. This experiment had been done in order to have a band of APP.

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