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REAL-TIME MULTIPLEX PCR ASSAY FOR INVESTIGATION OF THE KINESIN LIGHT-CHAIN 1 GENE A185C AND C406T MUTATIONS

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ABSTRACT

The kinesin is the main motor protein in the trafficking system of the mitochondria and other organelles. Genetic variants affecting the function of kinesin were earlier found to be associated with cognitive disturbances and neurodegeneration. A multiplex polymerase chain reaction (mPCR) method, allowing the simultaneous detection of the KNS2 A185C (rs_1054080) and C406T (rs_3742465) mutations has been developed. The new assay was validated by PCR-RFLP experiments: the proposed method and the PCR-RFLP analysis yielded identical results. This suggests the applicability of the mPCR assay for the investigation of KNS2 mutations in population studies.

KEYWORDS

kinesin; light chain; KNS2 mutations; multiplex PCR

1. INTRODUCTION

The kinesins are main cytoskeleton motor proteins whose function is to transport organelles within cells [2]. These are tetrameric proteins comprising 2 heavy chains (alpha chains, KNS1) and 2 light chains (beta chains, KNS2) [6]. The KNS1 chains provide the tubulin binding site and the ATPase domains, whereas the KNS2 chains are responsible for the specific attachment of the organelle to be moved by the kinesin tetramer. One special transport mediated by direct binding to the kinesin is the axonal moving of amyloid precursor protein carrying vesicles [7]. This transport is inhibited by overexpression of the *tau* protein [4], and this mechanism is enhanced by oxidative stress [10]. Thus, the amyloid precursor protein can be retained in the cells, thereby promoting the accumulation of amyloid β-peptides in the cytoplasm. Both the aggregation of the *tau* protein into abnormal filaments and the accumulated amyloid β plaques are specific for Alzheimer's disease (AD) [5].

Dhaenens et al. hypothesized that a KNS2 dysfunction plays a role in the pathogenesis of AD [3] and investigated the occurrence of three KNS2 polymorphisms in AD. Two of them, in the 5'UTR region (A185C and C406T), displayed no association, while the KNS2 G56836C mutation, located in intron 13 (mistyped by Dhaenens et al., and known as KNS2 G58836), exhibited an association with AD [3, 1]. This association for the KNS2 G56836C variant may result from an altered splicing effect [8].

Kinesins have been presumed to play a role in the pathomechanism of leukoaraiosis, which brings about a cognitive decline in a considerable proportion of the middle-aged and elderly [11]. In large population studies, the investigation of these polymorphisms via the recommended PCR-RFLP method [3] is laborious, expensive and time-consuming.

2. THE STUDY

A real-time PCR (LightCycler 1.5, Roche) assay with melting point analysis has been developed: KNS2 A185C (rs_1054080) and C406T (rs_3742465) mutations were investigated in multiplex PCR reaction. Primers described earlier [3], was modified for equal annealing temperatures which is necessary for the simultaneous amplification. Optimised primer and probe sequences and their concentrations are listed in Table 1.

Table 1. The sequences of the primers and probes

Primers/probes	Sequences	Cc (μM)
KNS2 A185C		
KNS185 for	AgCgggACTggCTggg	0.4
KNS185 rev	TCggCTgTgTgAggCACg	0.4
KNS185 probe	Flu-CCCCTCgCTggTgACTgCT-Ph	0.3
KNS185 anch	TgCggggCggTAgCTCCg-LCRed640	0.3
KNS2 C406T		
KNS406 for	TCCCTgTCCCgCTCCTCTTC	0.4
KNS406 rev	gACAAgAACCCgACCTgAACCTAgAAg	0.4
KNS406 probe	CCgCAACTCTgTCCCCATCCA-LCRed705	0.2
KNS406 anch	Flu-gCCTCCTTCCCggTTTggTCCCg-Ph	0.2

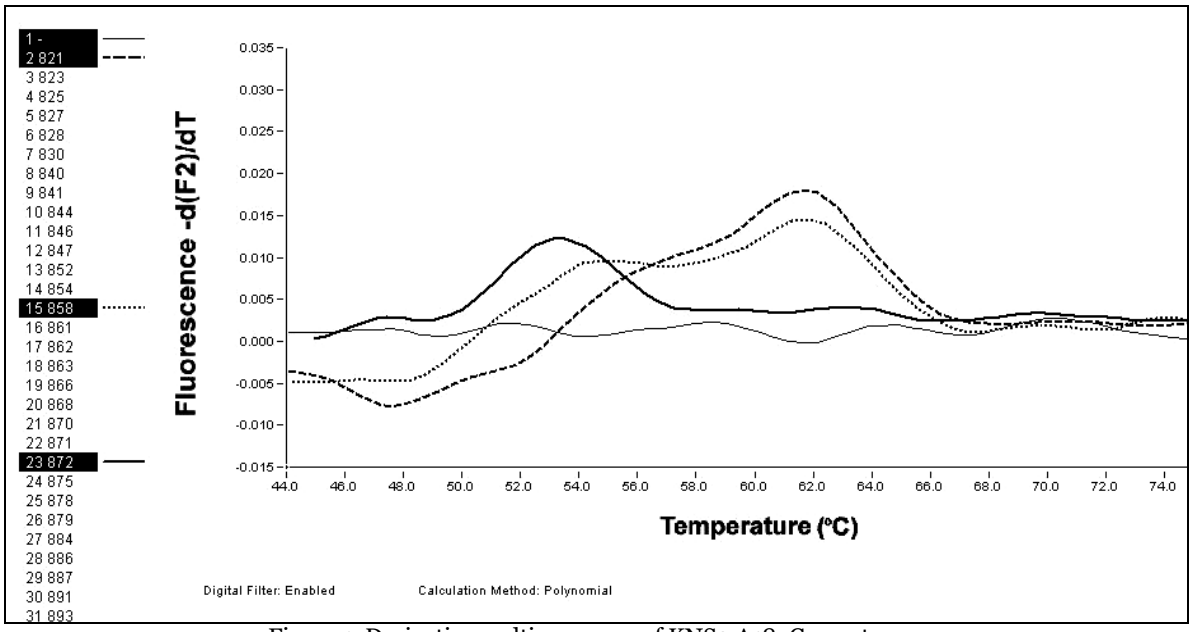

Figure 1. Derivative melting curves of KNS2 A185C genotypes.
(---- C/C, —— A/A, ····· C/A)

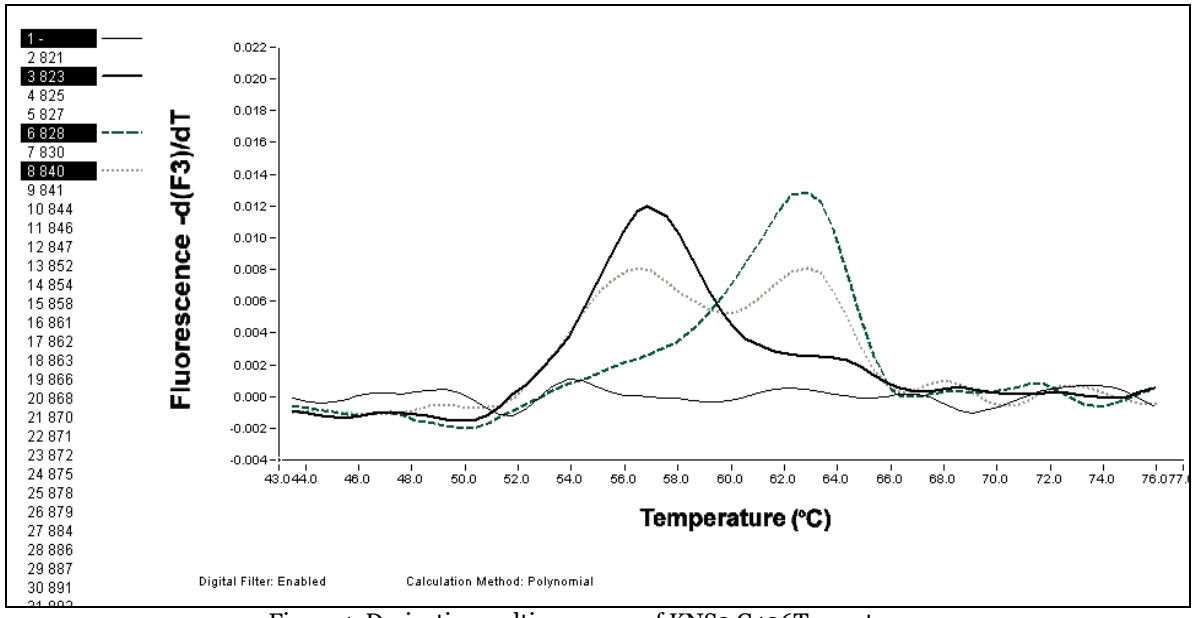

Figure 1. Derivative melting curves of KNS2 C406T genotypes.
(---- C/C, —— T/T, ····· C/T)

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The experimental protocol was the following: Genomic DNA was extracted from 200 μL of peripheral blood anticoagulated with EDTA [9]. All blood samples were kept at –20°C until DNA isolation. PCR was performed in disposable capillaries (Roche Diagnostics). The reaction (10 μL) contained 1 μL of DNA (40-80 ng), 0.4 μM of each of the primers, 1 μL of buffer (LightCycler DNA master hybridization probes 10X buffer, Roche), 0.4 μL of 25 mM $MgCl_2$ stock solution, 0.6 μL of DMSO and the probes (concentrations listed in Table 1). The PCR conditions were: initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation (95°C for 0 s, 20°C/s), annealing (60°C for 10 s, 20°C/s), and extension (72°C for 12 s, 20 °C/s).

The melting curve analysis consisted of 1 cycle at 95 °C for 10 s, 40 °C for 10 s, and then increase of the temperature to 75 °C at 0.2 °C/s. The fluorescence signal (F) was monitored continuously during the temperature ramp and then plotted against temperature (T). These curves were transformed to derivative melting curves [(-dF/dT) vs. T]. The KNS2 A185C mutation analysis was happened in the F2 channel (640 nm, Fig. 1.) and the C406T mutation analyses in the F3 channel (705 nm, Fig. 2.)

3. ANALYSIS AND DISCUSSION

The new mPCR assay and PCR-RFLP method yielded identical results (tested in 25 cases each): the melting temperatures and restriction fragment lengths are listed in Table 2. Among 251 control patient samples, the genotypes were KNS2 AA185: 16 (6.4%); KNS2 A185C: 104 (41.4%); KNS2 185CC: 131 (52.2%); KNS2 CC406: 143 (56.9%); KNS2 C406T: 94 (37.5%) and KNS2 406TT: 16 (5.6%). The allele frequencies are listed in Table 2. These data are comparable to those previously documented in Caucasian controls [3].

Table 2. Melting temperatures, RFLP lengths and allele frequencies of different genotypes. *In the original article [7], the isoschizomer *Bfa*I (New England Biolabs) was used. The restriction enzymes originated from Roche Diagnostics (Mannheim, Germany).

Alleles	Tm (°C)	RFLP (bp)	Allele frequency (%)
KNS A185C		*Mae*I*	
A	50.6	120+37	27.1
C	56.5	157	72.9
KNS C406T		*Mae*III	
C	63.0	167	75.7
T	57.0	132+35	24.3

4. CONCLUSIONS

The results obtained suggest the applicability of the new multiplex PCR assay for the investigation of KNS2 mutations in population studies without the disadvantages of the RFLP-based approaches.

REFERENCES

[1] Andersson ME, Sjolander A, Andreasen N et al.: Kinesin gene variability may affect tau phosphorylation in early Alzheimer's disease. Int J Mol Med 20, 233-239 (2007).
[2] Chernajovsky Y, Brown A, Clark J: Human kinesin light (beta) chain gene: DNA sequence and functional characterization of its promoter and first exon. DNA Cell Biol 15, 965-974 (1996).
[3] Dhaenens CM, Van Brussel E, Schraen-Maschke S, Pasquier F, Delacourte A, Sablonniere B: Association study of three polymorphisms of kinesin light chain 1 gene with Alzheimer's disease. Neurosci Lett 368, 290-292 (2004).
[4] Ebneth A, Godeman R, Stamer K, Illenberger S, Trinczek B, Mandelkow E: Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. J Cell Biol 143, 777-794 (1998).
[5] George HPS, Rossor M: Alzheimer's disease. Unravelling the disease process. Lancet 358, S1 (2001).
[6] Goldstein LS: Molecular motors: from one motor many tails to one motor many tales. Trends Cell Biol 11, 477-482 (2001).
[7] Kamal A, Stokin GB, Yang Z, Xia C, Goldstein LS: Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron 28, 449-459 (2000).

[8] McCart AE, Mahony D, Rothnagel AJ: Alternatively spliced products of the human kinesin light chain 1 (KNS2) gene. Traffic 4, 576-580 (2003).
[9] Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16, 1215-1218 (1988).
[10] Stamer K, Vogel R, Thies E, Mandelkow E, Mandelkow EM: Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. J Cell Biol 156, 1051-1063 (2002).
[11] Szolnoki Z: Pathomechanism of leukoaraiosis: a molecular bridge between the genetic, biochemical, and clinical processes (a mitochondrial hypothesis). Neuromol Med 9, 21-33 (2007).

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