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A New Rapid Method for Detecting Val103Ile and C-2745T Polymorphisms in the Melanocortin-4 Receptor Gene Using Multiplex Minisequencing

Nowa szybka metoda diagnostyki polimorfizmów Val103Ile i C-2745T receptora melanokortyny za pomocą minisekwencjonowania

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Abstract

Background. The melanocortin receptor (MC4R) regulates food intake and energy expenditure. Recent publications have shown the influence of MC4R polymorphisms (Val103Ile and C-2745T) on the level of physical activity.
Objectives. To present a novel, rapid method for detecting those polymorphisms using a minisequencing technique.
Material and Methods. The effects of Val103Ile and C-2745T polymorphisms were studied in 452 healthy men from Lower Silesia (Poland). Genomic DNA was obtained from whole blood using standard isolation methods. Multiplex polymerase chain reaction (PCR) and minisequencing was used to detect Val103Ile and C-2745T polymorphisms. Minisequencing products were separated by capillary electrophoresis. Alleles were analyzed using GeneScan 3.1.2 software.
Results. PCR and minisequencing conditions were optimized.
Conclusions. The minisequencing method may be utilized in a multiplex detection system. The technique was used to identify two polymorphisms in the *MC4R* gene. It was found that their frequency was concordant with the distribution reported in other populations (**Adv Clin Exp Med 2010, 19, 5, 573–577**).

Key words: melanocortin receptor, melanocortin, PCR, minisequencing, obesity.

Streszczenie

Wprowadzenie. Receptor melanokortyny (MC4R) jest jednym z elementów regulacji zarówno pobierania pokarmu, jak i wydatkowania energetycznego. Ostatnie publikacje wskazują na udział polimorfizmów tego genu w regulacji poziomu aktywności fizycznej.
Cel pracy. Opracowanie nowoczesnej, szybkiej i taniej metody diagnostyki polimorfizmów genu *MC4R*. Do tego celu wykorzystano technikę minisekwencjonowania.
Materiał i metody. W niniejszej pracy zbadano populację 452 zdrowych mężczyzn z terenu Dolnego Śląska. Genomowe DNA izolowano z leukocytów krwi obwodowej z użyciem standardowej metody kolumienkowej. W celu namnożenia materiału genetycznego użyto techniki multiplexPCR, a następnie określono polimorfizmy Val103Ile i C-2745T genu *MC4R* minisekwencjonowaniem. Produkty reakcji zostały rozdzielone z użyciem elektroforezy kapilarnej. Poszczególne allele zidentyfikowano oprogramowaniem GeneScan 3.1.2.
Wyniki. Zoptymalizowano warunki reakcji PCR oraz minisekwencjonowania.
Wnioski. Technika minisekwencjonowania służy do identyfikacji kilku punktowych polimorfizmów (SNP)/mutacji w jednej reakcji. Dzięki jej zastosowaniu uzyskano metodę do szybkiej analizy polimorfizmów genu *MC4R* z jednoczesnym znacznym ograniczeniem kosztów i czasu analizy (**Adv Clin Exp Med 2010, 19, 5, 573–577**).

Słowa kluczowe: receptor melanokortyny, melanokortyna, PCR, minisekwencjonowanie, otyłość.

The melanocortin system was discovered in the 1950s [1]. The first components of the system to be identified were the endogenous melanocortin agonists. Five isoforms of the melanocortin receptors and endogenous antagonists have subsequently been described [2]. The melanocortin system is extremely important for human energy homeostasis [3]. Melanocortin receptors activated by one or more melanocortins increase the intracellular cAMP concentration [4].

In the 1990s it was found that the melanocortin-4 receptor (MC4R) – one of the five identified melonocortin receptors – is involved in the mechanisms underlying obesity [5]. Recent publications have also shown that the MC4R polymorphism influences the level of physical activity in humans [6].

MC4R is composed of 332 aminoacids forming seven transmembrane helices linked to G-protein. Its sequence is homologous to the cannabinoid receptor [7]. MC4R is expressed mainly in the central nervous system [8]. According to Nature "the α-Melanocyte stimulating hormone (α-MSH) is the most relevant endogenous agonist for MC4R, whereas agouti-related protein is its natural antagonist". MC4R is involved in central energy homeostasis regulation and body composition; e.g., genetic disruption of MC4R induces obesity in mice [5]. Melanocortin receptor agonists have been found to inhibit food intake [9].

More than 70 MC4R polymorphisms are known, including missense, nonsense and frameshift mutations. Polymorphisms that impair MC4R function (many of the missense and all of the frameshift mutations) may influence obesity and the level of the physical activity [10].

The Val103Ile polymorphism is the most common variation in this gene. It associates with severe obesity, while the T/T variant of the C-2745T polymorphism (in the promoter region) is related to the level of physical activity [6].

Minisequencing is one of the methods based on polymerase chain reaction (PCR). It is used to detect single nucleotide polymorphisms or point mutations. The method entails incorporating a single fluorescently labelled dideoxynucleotide (ddNTP) at the 3' end of a special primer which is complementary to the sequence located one nucleotide before the examined polymorphic site. The incorporation of one of four ddNTPs labelled by different color dyes depends on the genotype. The products of the reaction are then separated by capillary electrophoresis. The color of the signal or signals obtained (one for homozygotes, two for heterozygotes) allows the incorporated ddNTP to be identified, and furthermore the complementary deoxynucleotide in the DNA sequence. If multilenght oligonucleotides are used, several different polymorphisms/mutations can be detected in one multiplex reaction. [11]

We want to present a novel, rapid technique for using the minisequencing method to detect two of the main MC4R polymorphisms that influence physical activity and obesity in humans.

Material and Methods

The effects of the Val103Ile and C-2745T polymorphisms were studied in 452 men living in the Lower Silesia region of Poland, (HALS Study) aged from 22 to 72 years, randomly chosen from the Regional Statistical Office's inhabitants database. All of them were of European descent.

Whole genomic DNA was obtained from blood leukocytes by standard isolating methods. Melanocortin receptor genotyping was performed by PCR and minisequencing.

Multiplex PCR

To amplify the 146-bp (Val103Ile) and 570 bp (C-2745T) fragments of the melanocortin receptor gene in a single reaction, a mix was used containing: forward Val103Ile primer (5'-GAATCTGCATTCACCCATGTACT-3'), reverse Val103Ile primer (5'-CAATATTCACTGTGAAACTCTGT-3'), forward C-2745T primer (5'-GGCATTTCTCCAAAGATTATTAC-3'), reverse C-2745T primer (5'-ACCTTGCTAATTTTTTGTAT-3'), 1x PCR buffer, 1.5mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 2 polymerase units (ROCHE), 200 ng genomic DNA and water up to 20 μl.

The DNA was denatured at 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 30 seconds. The final extension was at 72°C for 10 minutes. The size and quantity of PCR products were analyzed using 2.5% agarose electrophoresis.

Minisequencing

The amplified products (the 570-bp and 146-bp fragments) were purified from oligonucleotides and free dNTPs by SAP and ExoI treatment (Fermentas).

The minisequencing method was based on the incorporation of single fluorescence-labeled dideoxynucleotides to the 3' end of the oligonucleotide which was correctly paired to the specific template DNA fragment using a SNaPshot kit (Applied Biosystems). The SNaPshot reaction was carried out using oligonucleotides:

– C-2745T: 5'-TCATGAGGTCAGGAGATC-GAGACCA-3'

– Val103Ile: 5'-TGCTGGTGAGCGTTTCA-AATGGATCAGAAACCATT-3'

that ended just before the polymorphic sites. The SNaPshot reaction consisted of 25 cycles: denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 30 seconds.

Electrophoresis

The products (26 bp and 36 bp respectively) were purified from the free ddNTPs by SAP treatment and then analyzed using an ABI 310 sequencer (Applied Biosystems).

The samples were suspended in formamide and LIZ-120 was added; the mixture was denaturated for 4 minutes at 94°C and then cooled in ice (or 4°C).

We used POP-4 polymer in conjunction with the GS E5 Run Module to separate the SNaPshot products. All the data were gathered using dedicated software. The alleles were calculated using GeneScan 3.1.2 software (Applied Biosystems). The color peak for each extended minisequencing primer was scored for each sample.

Results

PCR Optimalisation

In the first stage, duplex PCR temperature conditions were set. For this purpose we performed PCR at a gradient annealing temperature of 50–60°C. The best amplification yield was obtained at 53°C (see Fig. 1). A combination of two SAP units and one ExoI unit was used to purify (25 µl) the PCR product from oligonucleotides and free dNTPs.

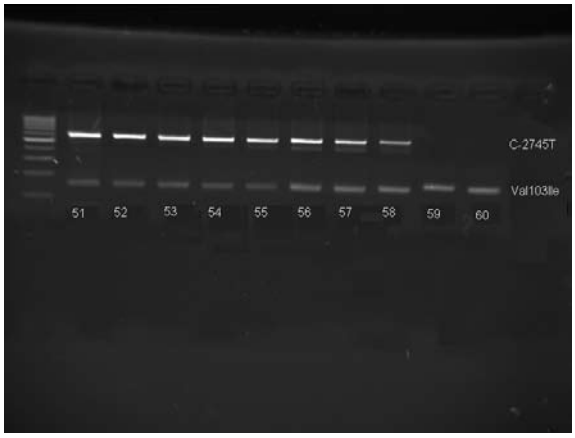

Fig. 1. Annealing temperature optimization for multiplex PCR with a set of primers for Val103Ile and C-2745T. DNA was isolated from the main author's peripheral blood

Ryc. 1. Optymalizacja temperatury przyłączania reakcji multiplexPCR z użyciem starterów komplementarnych do fragmentów Val103Ile oraz C-2745T

Minisequencing

The aim of using two oligonucleotides in the minisequencing reaction was to facilitate multiplex detection of the melanocortin polymorphisms. Two fluorophore-labeled products were obtained. The cytosine was in the position 2745 when 26 bp products presented yellow fluorescence (dTAMRA) and tymine – red (dROX). Analogous G was at position 103 when 36 bp products fluoresced blue (dR110) and a fluoresced green (dR6G). Typical results are shown in Fig. 2.

Table 1. Allele frequency of polymorphisms Val103Ile and C-2745T

Tabela 1. Częstotliwość alleli polimorfizmów Val103Ile i C-2745T

	C-2745T		Val103Ile
n	425	n	452
C/C	188	V/V	431
C/T	180	V/I	20
T/T	57	I/I	1
pC	0.65	pV	0.98
pT	0.35	pI	0.02
χ square	1.741567	χ square	2.104417
p	< 0.418624	p	< 0.349167

n – number of persons; C/C – dominant haplotypes; C/T – heterotypes; T/T – recessive haplotypes; V/V – dominant haplotypes; V/I – heterotypes; I/I – recessive haplotypes; pC, pT, pV, pI – allele probability.

n – liczba osób; C/C – dominujące haplotypy; C/T – heterotypy; T/T – recesywne haplotypy; V/V – dominujące haplotypy; V/I – heterotypy; I/I – recesywne haplotypy; pC, pT, pV, pI – prawdopodobieństwo alleli.

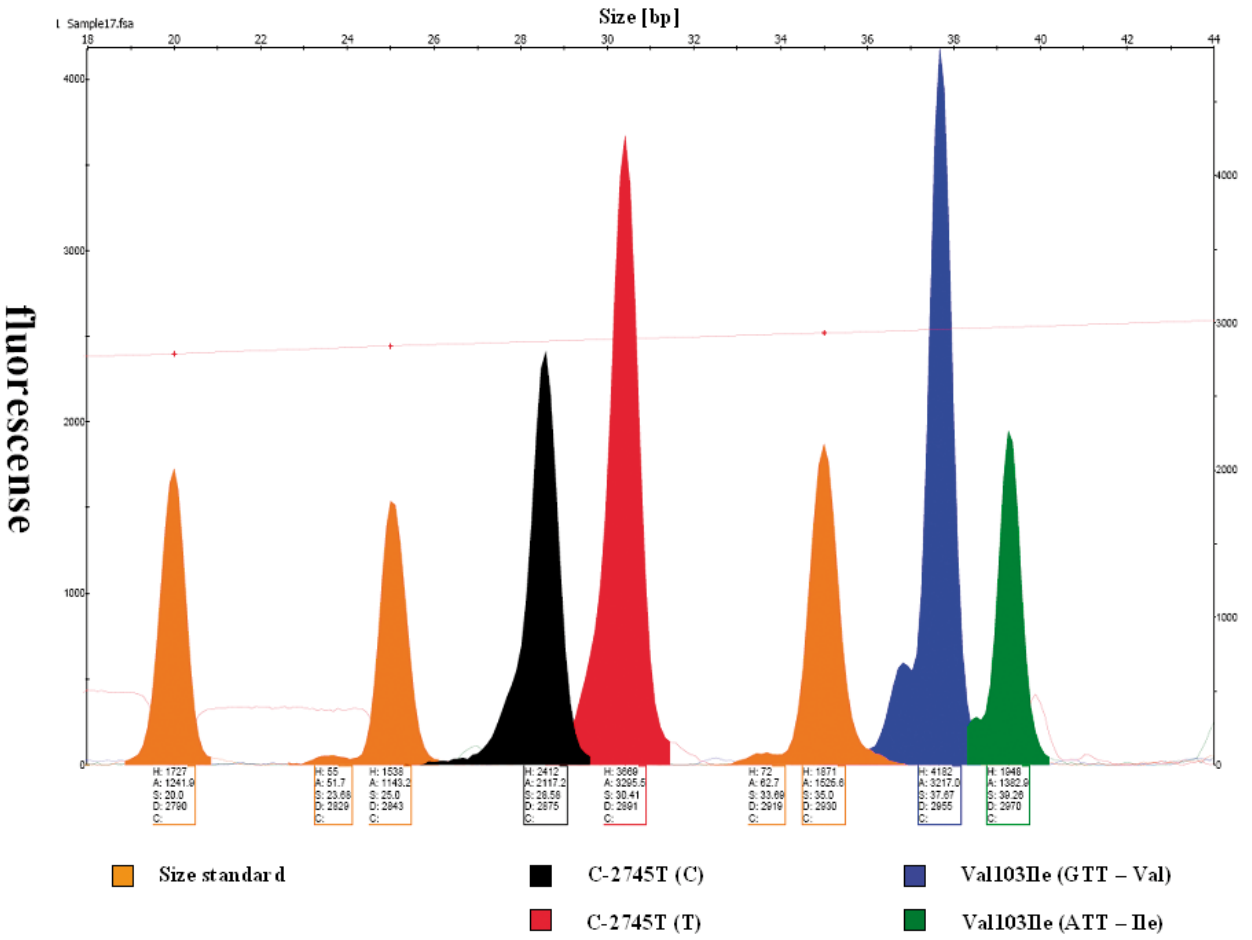

Fig. 2. Melanocortin receptor multiplex minisequencing results (Val103Ile and C-2745T genotyping). Orange – mass standard; green – A; black – C; blue – G; red – T

Ryc. 2. Wyniki genotypowania receptora melanokortyny reakcją minisekcjonowania. Kolory: pomarańczowy – standard masy; zielony – A; czarny – C; niebieski – G; czerwony – T

The method described was used to type the C-2754T polymorphism in 425 persons and the Val103Ile polymorphism in 452 persons. We found 188 dominant haplotypes, 57 recessive haplotypes and 180 heterotypes of the C-2745T polymorphism. Dominant haplotypes of the Val103Ile polymorphism were present in 431 subjects, recessive in one subject and heterotypes in 20 subjects (Table 1). Six probes (C2745T: dominant haplotypes, recessive haplotypes, heterotypes; and Val-103Ile: dominant haplotypes, recessive haplotypes, heterotypes) were confirmed by sequencing.

The smallest value of the matrix concentration usable for DNA estimation is 10 ng/µl of DNA. The reaction is repeatable for about 50 measurements of the same material.

Discussion

We have demonstrated that a multiplex minisequencing method can be successfully used to detect gene polymorphisms affecting physical activity and obesity.

Techniques detecting single nucleotide polymorphisms (mutations) are widely used in molecular research. Sequencing is a fundamental method for confirming information on polymorphisms in particular genes. Unfortunately, it is expensive and time-consuming, and thus difficult to perform in routine genetic diagnostics. Other PCR-based methods like PCR-RFLP, ACRS-PCR-RFLP, ASA-PCR and ASO-PCR are fast and inexpensive, but deliver indirect and usually insufficient information on polymorphisms [11].

Minisequencing is considered a direct method for assessing polymorphisms or point mutation (SNP). The idea of the method is to connect a particular ddNTP (marked by a fluorescent indicator) to a properly designed starter (primer). Identification of the product in the material as well as the presence of a fluorochrome color defines the type of polymorphism (mutation) in the investigated area [11].

One advantage of the minisequencing method is that it can be utilized in a multiplex detection system. The use of a kit of several starters (primers) of different lengths allows multiple polymorphisms to be identified simultaneously.

In our investigation, the minisequencing technique was used to identify two polymorphisms in the MC4R gene. The frequency we found for them was comparable to the distribution detected in other populations by other methods. The reliability of the technique we used is also confirmed by its repeatability and by analyses of the assay limits.

It is worth mentioning that the presented method gives highly reliable results at significantly reduced research costs.

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