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Correlations between polymorphisms in genes coding elements of dopaminergic pathways and body mass index in overweight and obese women

Związek między wybranymi polimorfizmami w genach kodujących elementy szlaku dopaminergicznego a współczynnikiem masy ciała u kobiet z nadwagą i otyłością

Marcin Sikora[1], Anna Gese[1], Ryszard Czypicki[1], Marcin Gąsior[1], Andrzej Tretyn[1], Jacek Chojnowski[2], Maciej Bieliński[3,5], Marcin Jaracz[3], Anna Kamińska[4], Roman Junik[4], Alina Borkowska[3]

[1]*Nicolaus Copernicus University Biotechnology Department, Toruń, Poland*
[2]*Department of Balneology, Collegium Medicum NCU, Ciechocinek, Poland*
[3]*Department of Clinical Neuropsychology, Collegium Medicum, NCU Bydgoszcz, Poland*
[4]*Department of Endocrinology and Diabetology with a Nuclear Medicine Laboratory, Collegium Medicum NCU, Bydgoszcz, Poland*
[5]*Jan Biziel's University Hospital Division of Vascular Diseases and Internal Medicine, Bydgoszcz, Poland*

Abstract

Introduction: Dopamine is considered to be crucial for food craving and intake, drug abuse and electrical brain stimulation. Increased levels of dopamine occur after energy intake in the dorsal striatum. In the ventral tagmental area, dopamine is responsible for motivation. There is a natural synaptic dopamine level, and as a result its activity is controlled by density of receptors, amount of released neurotransmitter, and defectiveness of re-uptake by specific transporters. In our study, we wanted to investigate if there is a correlation between mean BMI values and VNTR polymorphisms in *SLC6A3* (rs28363170) and *DRD4* genes.

Material and methods: Chosen gene fragments were amplified using polymerase chain reaction on the DNA template obtained from 506 women. The products of the reaction were electrophoresed and visualised in 3% agarose gel. The genotyping data was analysed with Kruskal-Wallis tests ($p < 0.05$).

Results: In the case of *SLC6A3,* statistically significant differences in mean BMI were found in the group of obese women ($p < 0.05$) but not for the whole population of women with normal weight or with overweight ($p > 0.05$). The mean BMI was higher for the SS genotype than for combined LL and LS genotypes. The difference in mean BMI values for variants of *DRD4* was significant for the whole studied population and in the obese group ($p > 0.05$), and the higher value was correlated with the presence of a variant with seven or more repeats of 48 bp motif.

Conclusions: When the two analysed polymorphisms were combined, the spread between the mean BMI values became greater than for single genes. This suggests that the effect on body mass of these two polymorphisms may combine and cause hypo-functionality of the dopaminergic reward system. **(Endokrynol Pol 2013; 64 (2): 101–107)**

Key words: *dopamine, SLC6A3, DRD4, obesity*

Streszczenie

Wstęp: Dopamina jest uważana za kluczowy związek w regulacji łaknienia, przyjmowaniu pokarmów, uzależnienia od leków oraz w stymulacji elektrycznej mózgu. Zwiększony poziom dopaminy obserwuje się po posiłkach w grzbietowej części prążkowia. W brzusznej części nakrywki dopamina odpowiada za motywację. Naturalny poziom synaptyczny dopaminy wynika z gęstości receptorów, ilości uwolnionego neurotransmitera oraz efektywności działania systemu wychwytu zwrotnego. W niniejszym badaniu poddano analizie korelację wartości BMI z polimorfizmem genu DRD4 oraz polimorfizmu SLC6A3.

Materiał i metody: Wybrane fragmenty genu były zwielokratniane przy pomocy reakcji łańcuchowej polimerazy (PCR, *polymerase chain reaction*) z DNA uzyskanego od 506 kobiet. Produkty zwielokrotnienia były poddawane elektroforezie. Wyniki genotypowania analizowano za pomocą testu Kruskal-Wallis ($P < 0,05$).

Wyniki: Analizując wyniki korelacji polimorfizmu SLC6A3 istotne statystycznie różnice w zakresie wartości BMI dotyczyły jedynie kobiet otyłych ($P < 0,05$), nie potwierdzając się wśród kobiet z prawidłową i nadmierną masą ciała. Średnia wartość BMI była wyższa w przypadku genotypu SS, niż w zakresie rozważanych łącznie genotypów LL i LS. Różnice średnich wartości BMI w kontekście polimorfizmu DRD4 były istotne w całej analizowanej populacji. Wyższa wartość BMI korelowała z obecnością wariantu 7 i więcej powtórzeń motywu 48 par zasad.

Wnioski: Prowadząc analizę łącznie korelacji z BMI dla obu polimorfizmów, istotność stawała się większa niż dla poszczególnych genów. Taka sytuacja może dowodzić, iż wpływ polimorfizów obu badanych genów mogą się kumulować i prowadzić do obniżenia działania dopaminergicznego układu nagrody. **(Endokrynol Pol 2013; 64 (2): 101–107)**

Słowa kluczowe: *dopamina, SLC6A3, DRD4, otyłość*

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Maciej Bieliński M.D., Ph.D., Department of Clinical Neuropsychology, Marii Curie-Skłodowskiej St. 9, 85–094 Bydgoszcz, tel./fax: 52 585 37 07, e-mail: bielinskim@gmail.com

Introduction

Obesity is a chronic non-infectious disease of complex aetiology. It is believed that environment as well as genetic variety may be important for its pathogenesis. Overweight and obesity are connected with many serious complications. Weight restriction results in multiple advantages, such as improvements of blood inflammatory markers [1]. Obesity is defined as an excessive accumulation of body fat due to positive energy balance. The most useful way to assess the prevalence of obesity is the body mass index (BMI) defined as one's weight in kilograms divided by the square of one's height in metres. According to the WHO, individuals with BMI ≥ 25 kg/m^2 are considered overweight and with BMI ≥ 30 kg/m^2 obese.

Dopamine (DA) is a crucial neurotransmitter in food intake control. The main centres of this process are located in the midbrain (VTA [ventral tagmental area] and SNpc [substantia nigra pars compacta]) and in the nucleus accumbens (NAcc) located in the ventral striatum (VS). Release of dopamine in VTA is responsible for the craving for food. Agonists which modify brain dopamine levels reduce further food consumption [2], while antagonists increase energy intake [3]. In SNpc, DA plays also the key role in stimulus-reward learning in a way that food and accompanying saliencies become considered as potential sources of reward [4]. The third role of dopamine is to stimulate the feeling of pleasure in the nucleus accumbens (in conjunction with the opioid and endocannabinoid signals) in response to energy intake, especially in the form of palatable food rich in carbohydrates and fat. Increased levels of this neurotransmitter can be observed after energy intake in the dorsal striatum NAcc [5].

There is a natural synaptic dopamine level, and as a result its activity is controlled by density of receptors, amount of released neurotransmitter, and defectiveness of re-uptake by specific transporters [6-8]. In our study, we focused on widely analysed polymorphisms in genes coding dopamine transporter (*SLC6A3*) and dopamine receptor D4 (*DRD4*).

Dopamine transporter (DAT), encoded by *SLC6A3* gene, is a protein responsible for DA re-uptake from synaptic cleft into pre-synaptic neuron, which in turn is the major means of termination of neurotransmission [9]. It consists of 12 transmembrane domains characteristic for neurotransmitter transporters dependent on Na^+ gradient (generated by Na^+/K^+ ATPase) and Cl^- ions [10]. The highest level of *SLC6A3* expression can be observed in dopaminergic neurons of VTA and substantia nigra (SN) [11]. These nerve cells project to the striatum, NAcc and prefrontal cortex [12]. In our study, we focused on the polymorphism rs28363170 where alleles differ in variable number of tandem repeats (VNTR) in 3'-untranslated region, with the most common variants being 9R and 10R repeats [13]. The 10R allele was correlated with increased expression of DAT in one study using the luciferase expression system [14] and with lowered in another [15]. It has also been demonstrated that allele 9R can be responsible for increased availability of DAT in the human striatum [16].

The human dopamine receptor D4 is coded by the *DRD4* gene, which is described as having a high number of polymorphisms [17]. In our study, we focused on 48-bp variable number of terminal repeats (VNTR) in third exon, encoding 3 intracellular loop. The number of repeats in this region of *DRD4* gene may vary from two to 11 repeats (2R-11R). Variants with 2R are the most common [18]. Furthermore, even the variants with the same length may differ significantly in nucleotide sequence [19]. It was suggested that 7R variant has inferior ability to reduce cyclic adenosine monophosphate (cAMP) compared to shorter forms [20]. The most significant connection of the genotype with phenotype was found for allele 7R and ADHD [21]. Polymorphisms of the *DRD4* gene are considered to be crucial in the pathogenesis of some psychiatric disorders. However, a study in a Polish population showed no correlations of DRD4 alleles and alcoholism [22].

Material and methods

Study population characteristics

Age of subjects was 18–84 years with mean 50.7 (SD ± 15.0). Subjects were divided into groups based on their body mass index (BMI; according to WHO recommendation). Mean BMI for the studied population was 33.4 (SD ± 10.0). Detailed data on BMI in different classes of weight is shown in Table I.

Samples collection and DNA isolation

Permission for research was granted by the Bioethics Committee of Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland. Women of European Caucasian origin, patients and volunteers, were recruited at the General and Endocrinological Surgery Ward and the Department of Endocrinology and Diabetology of Collegium Medicum, Nicolaus Copernicus University Hospital in Bydgoszcz and at the Cardiological Clinic of Non-public Health Care Institution CITOMED in Toruń. Written consent was obtained from all subjects. Patients and volunteers were measured and weighed with standard medical equipment. DNA was obtained from the peripheral blood leukocytes from 506 women using the rapid DNA isolation method [23] and stored at –20 °C until genotyping.

Table I. *Mean values and standard deviation for BMI and age in different classes of body mass*
Tabela I. *Średnie wartości wraz z odchyleniem standardowym dla BMI i wieku dla różnych klas masy ciała*

Body mass class (BMI range)	N	Mean BMI ± SD	Mean age ± SD
Normal (18.5–24.9)	106	22.3 ± 1.8	50 ± 15.8
Overweight (25–29.9)	114	27.1 ± 1.4	57.5 ± 12.9
Obese (≥ 30)	286	40.1 ± 8.2	48.3 ± 14.8
Obese I (30–34.9)	92	31.9 ± 1.4	55 ± 14.6
Obese II (35–39.9)	60	36.9 ± 1.5	45.4 ± 15.8
Obese III (≥ 40)	134	47.2 ± 6.3	45 ± 12.9

BMI — body mass index; SD — standard deviation

DNA analysis

Polymorphisms in all studied genes were analysed using the polymerase chain reaction and the agarose gel electrophoresis techniques.

The fragment of *SLC6A3* gene was amplified using the forward primer 5′-TGTGGTGTAGGGAACGGCCTGAG-3′ and reverse primer 5′-CTTCCTGGAGGTCACGGCTCAAGG-3′ [24]. The reaction mixture contained 5 pmol of each primer, 1.5 mM $MgCl_2$ (Fermentas), 0.2 mM dNTP (Fermentas), 1 x $(NH_4)_2SO_4$ *Taq* Buffer (Fermentas), 0.5 U of *Taq* polymerase (Fermentas), 100 ng of DNA and de-ionised H_20 to the final volume of 20 μL. The PCR programme included the following steps: initial denaturation (5 min/95°C), 30 cycles of denaturation (1 min/95°C), annealing (1 min/62°C) and elongation (1 min/72°C), and a final step of elongation (2 min/72°C). The PCR product was electrophoresed in 2% agarose gel with ethidium bromide (10 μg/mL) and visualised under UV light. The visible bands corresponded to the S allele (443 bp) and/or the L allele (483 bp) depending on genotype.

The PCR products on the matrix of *DRD4* gene were obtained using DRD4F 5′-GCGACTACGTGGTCTACTCG-3′ and DRD4R 5′-AGGACCCTCATGGCCTTGC-3′ primers [17]. The reaction mixture contained 5 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 mM of dATP, dCTP and dTTP, 0.1 mM of dGTP (Fermentas), 0.1 mM of 7-deaza-GTP, 1 x $(NH_4)_2SO_4$ *Taq* Buffer (Fermentas), 0.5 U of *Taq* polymerase (Fermentas), 50 ng of DNA, 10% DMSO (Sigma) and de-ionised H_2O to the final volume of 20 μL. The PCR protocol included the following steps: initial denaturation (10 min/94°C), 30 cycles of denaturation (1 min/95°C), annealing (1 min/57°C) and elongation (1 min/72°C), and final extension (2 min/72°C). PCR products were electrophoresed and visualised under UV light. Their lengths were 379 bp in the case of 2R, 427 bp for 3R, 475 bp for 4R, 523 bp for 5R, 571 bp for 6R and 619 bp for 7R. The longer products were defined as 7R+.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was analysed using Pearson χ^2 test ($p < 0.05$) with OOoStat package for OpenOffice.org Calc and R v 2.12.0. [25]. The statistical significance of differences in mean BMI depending on genotype for study subjects were assessed by Kruskal-Wallis test ($p < 0.05$) implemented in the OOoStat package and R v 2.12.0. Analysis was performed for all women (A), control group (C), overweight group (OW), and obese in general (O) for two genes separately. In case of combined genotypes of *SLC6A3* and *DRD4,* genes analysis was performed separately for the whole population (WP) and for women with common (frequency > 5% in WP) *DRD4* alleles (CA).

Results

Genotype frequencies were in Hardy-Weinberg equilibrium for both polymorphisms and for each analysed sub-group (control, overweight and obese).

SLC6A3

Significant difference in mean BMI values between genotypes was observed in the case of obese ($p = 0.033$) under the assumption that allele L dominates. The mean BMI was higher for the SS genotype (43.6 ± 6.8) than for combined LL and LS genotypes (39.9 ± 8.2). There were no differences in BMI between the control and overweight groups. Details are shown in Table II.

DRD4

The allele frequencies in the whole population were: 2R — 0.090 (N = 91), 3R — 0.028 (N = 28), 4R — 0.695 (N = 702), 5R — 0.005 (N = 5), 6R — 0.002 (N = 2), 7R — 0.164 (N = 166) and 7R+ — 0.016 (N = 16). When only women with the most common variants were studied, analysis of the difference in the mean BMI between genotypes showed that there is a correlation between number of repeats and body mass index ($p = 0.000$). Women with the 2R/2R genotype had the lowest BMI (24.7 ± 2.8; N = 5), while those with 7R/7R had the highest (54.6 ± 2.7; N = 14). For other genotypes, the mean BMI values were: 2R/7R — 27.5 ± 0.7

Table II. *Differences in BMI between genotypes for SLC6A3 and DRD4*
Tabela II. *Różnice w BMI między genotypami dla SLC6A3 i DRD4*

DAT				DRD4			
DAT Genotype	**N**	**Mean BMI**	**S.D.**	**DRD4 Genotype**	**N**	**Mean**	**S.D.**
Whole population				Whole population			
LL+LS	479	33.3	9.9	LL+LS	168	34.9	10.7
SS	27	35.9	11	SS	338	32.6	9.6
	p = 0.228				p = 0.029*		
Control				Control			
LL+LS	101	22.3	1.7	LL+LS	28	22.5	1.5
SS	5	21.5	2.4	SS	78	22.1	1.8
	p = 0.375				p = 0.374		
Overweight				Overweight			
LL+LS	108	27.0	1.3	LL+LS	69	25.3	2.7
SS	6	27.5	1.8	SS	151	24.4	2.9
	P = 0.551				p = 0.351		
Obesity				Obesity			
LL+LS	270	39.9	8.2	LL+LS	99	41.7	8.8
SS	16	43.6	6.8	SS	187	39.3	7.7
	p = 0.033*				p = 0.025*		

N — number of patients with each polymorphism

(N = 19), 2R/4R – 29.6 ± 10.4 (N = 248) and 4R/7R 39.3 ± 4.6 (N = 114).

The mean BMI values for the genotypes with at least one 7R allele or 7R+ (L) were compared to those with shorter alleles (S). The differences between them were significant in the A ($p = 0.029$) group and in the O group ($p = 0.025$). Genotypes with one or more L allele had higher mean BMI (34.9 ± 10.7 for all women and 41.7 ± 8.8 for obese) compared to those with S (32.6 ± 9.6 and 37.6 ± 7.7 respectively) as shown in Table II.

SLC6A3 and DRD4

Analysis of combined polymorphisms was performed separately for the whole population and for women with the aforementioned common *DRD4* alleles. Similarly to the single polymorphisms analysis, in the case of the whole population and sub-population (Table II)with the most common *DRD4* alleles (Table III), the differences in mean BMI values were the most significant in the obese group ($p = 0.027$ for WP and $p = 0.010$ for CA). In the CA sub-population, the mean BMI values differed also for A ($p = 0.038$). Details are shown in Table IV.

Discussion

Our results show that polymorphisms in *SLC6A3* may influence BMI in the case of obese women. Higher BMI in this group was correlated with SS genotype. Data on the influence of VNTR polymorphism of *SLC6A3* gene on dopaminergic system function and eating disorders is inconsistent. Genotypes LS and SS are usually grouped together and compared to LL due to allele S rarity. In our study, the greatest differences were observed between SS and combined LL+LS in obese women, but not in overweight or the control group. This may be due to the cumulative effect of changes in function and activity of other elements of dopaminergic system. Obtained results are somehow consistent with the study of 90 Japanese women with diagnosed eating disorders (ED) compared to 115 healthy women with stable body weight, where frequencies of the short allele were higher in the first group and associated with ED [26]. It has been suggested that DAT activity in the striatum is higher in subjects with allele S [16, 27]. In this case, the explanation of higher BMI in patients with genotype SS would be that faster removal of dopamine from the synaptic cleft may lead to impaired stimulation of dopamine receptors in the striatum and nucleus accumbens in its ventral part in particular. The NAcc is responsible for rewarding food ingestion, and food richer in carbohydrates and fat (or higher amounts of it) may be needed to exert the same effect on the reward centre as in the subjects with genotype LS or LL. On the other hand, both lack of influence of L or S allele on the risk of obesity [28] or opposite dependence have been reported. For example, the results of a study on 88 smok-

Table III. *Differences in BMI between genotypes for SLC6A3 and DRD4 for the part of studied population with the variants of DRD4 with frequency higher than 5%*

Tabela III. *Różnice w BMI między genotypami dla genów SLC6A3 oraz DRD4 dla części populacji posiadającej allele DRD4 występujące z częstością wyższą niż 5%*

DAT				DRD4			
Genotype	**N**	**Mean BMI**	**S.D.**	**Genotype**	**N**	**Mean**	**S.D.**
Whole population				Whole population			
LL+LS	434	33.1	11.0	L	147	35.1	10.7
SS	24	37.2	9.8	S	311	32.5	9.4
	p = 0.067				p = 0.023*		
Control				Control			
LL+LS	94	22.2	1.8	L	23	22.4	1.7
SS	3	20.2	2.4	S	74	22.0	1.9
	p = 0.091				p = 0.460		
Overweight				Overweight			
LL+LS	95	27.0	1.4	L	36	27.2	2.32
SS	6	27.5	1.8	S	65	27.0	1.4
	p = 0.590				p = 0.652		
Obesity				Obesity			
LL+LS	245	39.7	8.1	L	88	41.6	9.0
SS	15	44.4	6.2	S	172	39.1	7.5
	p = 0.0085**				p = 0.031*		

N — number of patients with each polymorphism

ers by Epstein et al. [29] showed that the S allele may be correlated with lowered energy intake. In another study on 2,364 participants, the protective effect of the S allele on overweight and obesity was reported, though no data on differences between men and women, nor mean BMI values for the aforementioned genotypes, were described [30], which makes the data difficult to compare. In another study on 1,551 participants, the LL genotype was associated with increased high-calorie food intake in females [31].

The influence of *SLC6A3* VNTR is debated. Apart from claims that S or L variant has higher expression, there is a suggestion that this polymorphism is in linkage disequilibrium with other functional polymorphisms which are in fact responsible for the observed associations with different phenotypes [32].

Our study on *DRD4* VNTR conducted for the women with the most common alleles (2R, 4R and 7R) indicated that 2R/2R homozygotes had the lowest mean BMI value, while the carriers of two 7R alleles had the highest. When the alleles were clustered to the L and S groups, higher values of mean BMI could be observed when at least one copy of the L allele was present, especially in the obese sub-group as in the case of the *SLC6A3* gene. The fact that allele 7R was associated with blunted response to dopamine may explain why subjects with this allele had higher mean BMI [33]. Our results are consistent with those reported by Levitan et al. who claimed that 7R allele promotes weight gain in women with seasonal affective disorder [34] and with bulimia nervosa [35] as they are susceptible to binge eating. The interaction between *DRD4* 7R and *BDNF* 66Met alleles has been shown to contribute to weight gain in women with bulimia nervosa [36]. In addition, the 7R allele is connected with attention deficit hyperactivity disorder (ADHD) or attention deficient disorder (ADD) [37] and it has been stated that children with ADHD have a higher risk of obesity development [38-39]. In the case of ADHD, as well as obesity dopamine depletion and receptors, hypo-functionality may lead to decreased striatal activity of the dopaminergic system, which in turn contributes to deficient inhibitory control of feeding, aversion to delay of food ingestion leading to fast-food consumption, and compensation of lack of reward in the striatum involved in food- and sex-related activities.

The differences in BMI for genotypes of analysed genes, though significant, were not great and thus aforementioned single polymorphisms cannot be treated as crucial for development of obesity. However, when both analysed genotypes were combined, the spread between the highest BMI value for SS/L geno-

Table IV. *Comparison of mean BMI for combined polymorphisms. The table on the left shows the results for the whole population (WP) and on the right for the sub-population with the variants of DRD4 with frequency higher than 5% (CV)*

Tabela IV. *Porównanie wartości BMI dla kombinacji polimorfizmów. Po lewej stronie podano wyniki dla całej populacji (WP), a po prawej dla części populacji posiadającej allele DRD4 występujące z częstością wyższą niż 5%*

DAT/DRD4 (WP)				DAT/DRD4 (CA)			
Genotype	**N**	**Mean BMI**	**S.D.**	**Genotype**	**N**	**Mean**	**S.D.**
Whole population				Whole population			
LL+LS/L	157	34.8	10.6	LL+LS/L	136	34.9	10.7
LL+LS/S	322	32.2	9.5	LL+LS/S	298	32.3	9.3
SS/L	11	37.2	12.0	SS/L	11	37.2	12.0
SS/S	16	35.0	10.6	SS/S	13	37.0	10.6
	p = 0.111				p = 0.038*		
Control				Control			
LL+LS/L	26	22.7	1.4	LL+LS/L	21	22.6	1.6
LL+LS/S	75	22.1	1.8	LL+LS/S	73	22.1	1.9
SS/L	2	21.0	2.9	SS/L	2	20.9	2.9
SS/S	3	21.8	2.7	SS/S	1	18.7	-
	p = 0.517				p = 0.230		
Overweight				Overweight			
LL+LS/L	39	27.7	1.3	LL+LS/L	37	27.1	1.4
LL+LS/S	69	26.9	1.3	LL+LS/S	61	27.0	1.3
SS/L	2	27.9	1.6	SS/L	2	27.9	1.6
SS/S	4	27.2	2.1	SS/S	4	27.2	2.1
	p = 0.728				p = 0.885		
Obesity				Obesity			
LL+LS/L	92	41.4	8.9	LL+LS/L	81	41.4	9.1
LL+LS/S	178	39.1	7.7	LL+LS/S	164	38.9	7.4
SS/L	7	44.6	7.5	SS/L	7	44.6	7.5
SS/S	9	42.8	6.6	SS/S	8	44.3	5.3
	p = 0.027*				p = 0.010*		

N — number of patients with each polymorphism

type and the lowest for LL+LS/S was greater than for single polymorphisms. This shows that the effects of polymorphisms in *SLC6A3* and *DRD4* may synergise and result in impaired dopaminergic signalling.

There are two main models explaining how dopaminergic system impairment may lead to overweight and obesity. The first proposes hyper-response of reward system to food intake, resulting in overeating in a way similar to the development of drug addiction [40-41]. The second assumes that reward processing is impaired and overfeeding is a way of compensating for the deficiency of dopamine signalling [42].

We believe that the results of our study support the theory of obesity as a result of decreased stimulation of reward centre resulting from DRD4 hypo-functionality and increased DAT activity.

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