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Comparative Analysis of Phenylalanine Hydroxylase Mutations Spectrum in Novosibirsk and Kemerovo regions of Western Siberia, Russia

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Abstract

Results of phenylalanine hydroxylase (PAH) locus molecular genotyping for 115 phenylketonuria (PKU) patients and their family members from Novosibirsk and Kemerovo regions of Western Siberia are presented. The direct exons and adjacent introns regions sequencing was used to identify PKU-associated mutations. Mutations typical for Europe (p.R158Q, p.R252W, p.P281L, IVS10-11G>A, p.R408W, IVS12+1G>A) and typical for South-Eastern Asia and Turkey (p.R261Q и p.R243Q) were identified as well as a bunch of rare mutations (IVS2+5G>A, p.R155H, p.Y168H, p.W187R, E221_D222>Efs, p.A342T, p.Y386C, IVS11+1G>C). The p.R408W mutation was prevailing. Mutations spectrum for Novosibirsk region appeared to be more diverse than one for Kemerovo region.

Keywords: phenylketonuria, PKU, phenylalanine hydroxylase, PAH, genotype, phenotype.

Introduction

Phenylketonuria (PKU; MIM 261600) is a severe genetic disorder caused in most cases by the lack of phenylalanine hydroxylase (PAH; EC 1.14.16.1) activity leading to a failure in phenylalanine (Phe) to tyrosin (Tyr) conversion [1]. Accumulation of phenylalanine and toxic alternative pathways byproducts like phenylpyruvate, vinylacetate, phenyllactate, phenylacetylglutamine results innumerous symptoms including mental retardation. The lack of phenylalanine hydroxylase activity is commonly caused by the mutations in the corresponding 90 Kb long gene (*PAH*) located in chromosome 12 long arm segment q22-q24. Gene includes 13 exons encoding 451 a.a. protein [2]. The trait has autosomal recessive inheritance. There are more than 800 types of PKU-associated mutations in *PAH* locus known up to date and the amount is steadily increasing (http://www.pahdb.mcgill.ca). PKU is one of the most common genetic disorders with average frequency estimated as 1 per 10000 newborns. It is even more common in Western Siberia – 1 per 7000 newborns [3].

The present study was aimed to assess the spectrum of PKU-associated *PAH* gene mutations in PKU patients of Western Siberia and to compare mutations spectra of two different regions:

Novosibirsk region and Kemerovo region. The comparative analysis could provide the insights for understanding of the genetic structure and genetic history of populations. The precise identification of PKU-associated mutations types is important for providing personalized treatment and family planning for PKU-patients and their families involved in the study.

Materials and Methods

Patients

The cohort studied was composed of 115 unrelated PKU patients aged 5 years or less from Western Siberia, Russia. The PKU diagnosis was primary established by neonatal biochemical tests during years 2005-2013. Blood Phe concentration was assessed at day 3 or 4 after birth, and, if found elevated, the test was repeated later to confirm the diagnosis. Only patients with blood Phe levels of 120 microM or more were included in the studied cohort. The mutations types and inheritance was further confirmed by genotyping of patient's parents and sibs. Of total 115 patients studied 67.6% were residents of Novosibirsk region and 32.4% were residents of Kemerovo region.

Methods

The Phe concentration was assessed via fluorescent analyser Delphia-Victor (Perkin Elmer, Finland) according to manufacturer instructions. DNA was isolated from blood nuclear cells and purified by peptides precipitation in the presence of NaCl according to [4]. Exons and adjacent introns regions were PCR-amplified as 13 separate amplicons. PCR reaction mix of 40 mkl contained the following: 65 мМ Tris:HCl (pH 8.9), 16 мМ $(NH_4)_2$ SO_4, 1.5 мМ $MgC1_2$, 0.01% Tween-20, 10 мМ 2-mercaptoethanol, 0.1 mkM dNTP, 0.2 mkM oligonucleotide primers, 50-100 ng of genomic DNA, 2 u.a. Taq DNA pol (ICBFM SD RAS, Russia). PCR products amount and size were confirmed by agarose gel electrophoresis. Reaction mix for Sanger reaction of total volume 30 mkl contained 0.25-0.5 pmole of PCR product, 10 pmole of oligonucleotide primer, was done in 1 mkl of BigDye v.3.1 reagent and 6 mkl 5x sequencing buffer from BigDye Cycle Terminators Sequencing Kit (Applied Biosystems, UK). Cycling conditions for Sanger reaction were: 96°C for 1 min followed by 38 cycles 3 steps each: 98°C for 10 sec; 50°C for 5 sec; 60°C for 4 min. Unincorporated dyes and low M.W. components were removed via CentriSep spin columns (Princeton Separations, USA) according to manufacturer instructions. Purified Sanger reaction products were analyzed on ABI3130xl Genetic Analyser (Applied Biosystems, USA) in SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia).

The homozygosity level of alleles was assessed using equation $j=\Sigma f_i^2$, where f_i is allele frequency for allele i, as described in [5].

Results

We have identified 32 PKU-associated mutations types in the cohort studied, 86.7% of which being missense mutations, 9.5% - splicing mutations and 1.4% - deletions (see Table 1 for mutations types and frequencies). Vast majority of the mutations (55.8%) were in hemizygous state. Mutations were distributed over almost all of 13 PAH gene exons save exons 8 and 9. We also have found 7 neutral polymorphic sites: IVS1+62C>T, IVS1+134A>G, IVS2+19T>C, IVS3-22C>T, IVS4+47C>T, IVS5-54G>A, p.Q232Q.

Despite the relatively high number of identified mutations types only six of them (p.R408W, p.P281L, p.R261Q, p.R158Q, p.Y414C и IVS10-11G>A) account for more than 80% PKU-associated alleles. Relatively high diversity of mutation types could be accounted for intense migrations during contemporary population formation as well as it's highly mixed ethnic nature.

Homozygosity index for the cohort studied was 0.38 which is comparable with ethnically heterogenous Northern and Eastern Europe populations. For comparison we calculated homozygosity indexes from the literature data on mutations frequencies for some European countries: 0.58 for Latvia [6], 0.55 for Lithuania [7], 0.31 for Czech Republic [8], 0.38 for Poland [9], 0.38 for Iceland [10], 0.20 for Denmark [11]. On the contrary, calculated homozygosity indexes for more genetically homogenous Asian appeared to be much lower: 0.12 for Japan [12], 0.051 for Korea [13], 0.043 for China [14].

In our study we compared PKU-associated mutations spectra for two vast and highly populated regions of Western Siberia. Novosibirsk region situated in the middle of Eurasia and almost in the middle of Russian Federation on the South-East part of the West Siberian Lowland, one of the greatest plains in the World. The region is 178 thousands square kilometers vast which is

1% of Russian Federation territory. On 2010 it was populated by 2.66 million inhabitants representing 1.87% of Russian Federation population. Ethnically region population consists of mostly Russians (93.1%) with some Germans (1.2%), Ukrainians (0.9%), Tatars (0.9%) and some other nationalities (3.9% in total). Kemerovo region occupies the branches of Altay and Sayan Mountains at south-east of Western Siberia. It extends for 95.5 thousands square kilometers (4% of western Siberia and 0.56% of Russian Federation) and gives home for 2.76 million residents (1.87% of Russian Federation population) being the most dense populated part of Siberia. Russians also represent the majority of the region population (93.7%). Other ethnic groups present are also Tatars (1.5%), Germans (0.9%), Ukrainians (0.8%) and some other nationalities (4.1% in total).

Seventy nine unrelated PKU patients aged under 2 years participated in the study in Novosibirsk region. We were able to identify both PKU-associated *PAH* gene mutations in 75 of participants (94.9% of the cohort). Most widespread genotype p.R408W/p.R408W was identified in 30 patients (37.9% of the cohort); the next common genotype appeared to be p.R261Q/p.R408W identified in 9 patients. Missense mutation p.R408W appeared to be prevailing allele with allele frequency 63.6%. Only two other mutations (p.R261Q and p.R158Q) scored more than 5% allele frequency each. Several mutations (p.P281L, p.Y414C, IVS12+1G>A, IVS10-11G>A, IVS4+5G>T, p.L48S, p.R261X) had allele frequency from 1% to 3%. All other identified mutations were present as single cases. The notable feature of the region mutations spectrum was the discovery of several mutations (IVS2+5G>A, p.E390G, p.A403V, IVS1+5G>T, p.S349P) described as very rare or single cases in the populations of initial mutation discovery.

The cohort of Kemerovo region residents consisted of 37 patients and both PKU-associated *PAH* gene mutations were identified in 34 of them (91.9%). Mutation p.R408W was again the most prevailing (56.2% allele frequency) being found in homozygous state in 13 patients and in hemizygous with other mutations in another 15 patients. Only two mutations (p.Y414C and IVS10-11G>A) appeared to have allele frequency above 4%, two other rare mutations (p.R243Q and p.R155H) were discovered in one patient each but in homozygous state. All other identified alleles were present in just one occasion.

Discussion

Based on *PAH* gene mutations identification by the DNA sequencing of the corresponding loci we compared the PKU-associated mutation spectra for Novosibirsk and Kemerovo regions - two major districts of Western Siberia. As the possible factors playing role in this spectra formation one might regard the migration processes during the formation of contemporary regions populations. The initial development of Siberia by pioneers moving towards the Pacific coast was performed mainly by two social groups: developers (hunters, traders, manufacturers), capable for claiming of vast untouched territories for living and commercial use, and refugees expelled by authorities or life hardships into far previously unpopulated territories [15, 16]. Recently (since economic crisis of 1990-th) Novosibirsk had become the biggest migrations assimilation and redistribution center, where ample migrations streams of Russian-speaking people from Kazakhstan and former soviet Middle Asia republics were heading to. The natural conditions in the region are more favorable for housing and agriculture than in the other vast Siberian and Far East territories [17]. Contemporary Kemerovo region is one of the most industrial regions in Russia Federation [18], which population was also in significant part formed by active migration, particularly from the European part of the Soviet Union in the beginning and the middle of 20th century. The above mentioned migration flows could account for high polymorphism of PKU-associated mutations in Novosibirsk and Kemerovo regions.

The PKU-associated mutations spectra in Novosibirsk and Kemerovo regions share several common features. In both regions p.R408W absolutely dominates like in many European populations (76.0% in Latvia [6], 66.6% in Ukraine [19], 55.0% in Poland [9], 42.1% in Czech [8]. The next common mutation p.R261Q (13 hemizygous cases in Novosibirsk region and 3 – in Kemerovo region) is known to be wide spread in Switzerland and North Italy [10], Portugal [20] and Turkey [21]. The presence of p.R261Q could indicate the presence of Turkic alleles possibly introduced during Kipchak military tribes invasions [22]. The p.R158Q mutation frequency is below 4% in both regions. In many European populations this mutation is more common with frequencies from 5% to 10% [10]. The mutation p.P281L (3 hemizygous cases in each Novosibirsk and Kemerovo regions) is known to be common in South Europe [23 – 25] and to prevail in Iran,

Spain, Portugal, Germany and Poland [26 – 29]. The splice mutation IVS10-11G>A (also 3 hemizygous cases in each Novosibirsk and Kemerovo regions) is also spread in South Europe [23, 30 – 32]. Common for Scandinavia countries like Sweden and Denmark [10] missense mutation p.Y414C was also identified both in Novosibirsk and Kemerovo regions. All the above mentioned mutations (p.R408W, p.R261Q, p.R158Q, p.P281L, IVS10-11G>A, p.Y414C) present in both Novosibirsk and Kemerovo regions account totally for 80% of PKU-associated alleles.

The rest of mutations spectrum is quite different for Novosibirsk and Kemerovo regions. Generally the mutations diversity in Novosibirsk region is much higher than in Kemerovo region.

Many rare mutations were identified in Novosibirsk region patients, for instance rare splice mutations: IVS4+5G>T, previously described in Poland (9), IVS2+5G>A - initially discovered in Germany [33], IVS2-13T>G first reported in Italy [23] and IVS1+5G>T discovered in Denmark [33]. Three single cases of deletions S16>XfsX1, IVS2+1delG, D222>STOP were also found in Novosibirsk region patients. Low frequency (less than 2%) mutations were also present in Novosibirsk region cohort in in quite a variety: p.L48S, p.R243Q, p.R261X, p.R243X, p.E280K, p.E390G, p.A403V, p.P407L, p.R408Q (see Table 1).

The mutations spectrum for Kemerovo region was not so diverse. We identified single cases of splice mutation IVS11+1G>C first mentioned in 1995 in Indian patient [34] and deletion E221_D222>Efs single cases described in Germany and Denmark [35]. We also found rare mutation p.Y386C, previously described in single occasions in USA, Ireland and Italy [5, 36 and 37]. Rare mutation p.R243Q was identified in homozygous state in a patient from Kemerovo region, being probably the result of the marriage of close relatives. Mutations of moderate frequencies (2-3%) in Kemerovo region were presented by p.R68S, p.R155H, p.Y168H, p.R243Q, p.R243X, p.A342T, p.Y386C, p.Y414C (see Table 1). Common for European population's mutation p.R252W was found in a single occasion in Kemerovo region, but not in Novosibirsk region.

Data on alleles (Table 1) and genotypes (Table 2) frequencies suggest strong influence of genes flows from Eastern (IVS4+5G>T, IVS2+5G>A, IVS2+5G>C, S16>XfsX1, D222>STOP, p.A403V, p.P407L), South (IVS2-13T>G) and Western (p.S349P, p.E280K) Europe for Novosibirsk region genes pool formation with significant income from Turkey (p.R261Q) as well. In Kemerovo region not only mutations of European origin were identified but some of South-Eastern Asia origin as well: p.R243Q and p.R155H. The mutation p.R243Q, discovered in homozygous state, is known to be common in Japan, Korea and China (18% in Chinese population) [38].

When comparing our data on PKU-associated PAH gene mutations diversity with the similar data for other regions [39] of Russian Federation one could notice general increase of rare mutations diversity by a price of the most common mutations (p.R408W, p.P281L) share when moving from West to the East. This tendency is particularly notable in Novosibirsk region, probably being a result of intense migrations flows during the area population formation especially in 20th century. For instance, migration income in Novosibirsk region during seven years from 2000 till 2006 reached more than 50 thousands with Kazakhstan, Uzbekistan, Kirgizia, Latvia, Moldova, Germany and Israel being the main sources of migration. During the same seven years total migrants to Kemerovo region reached more than 30.6 thousands. More than a half (52%) migrated to Kemerovo region from Kazakhstan, 35% migrated from Middle Asia (Kirgizia and Uzbekistan) and Ukraine, the rest 13% came from other ten CIS (Commonwealth of Independent States) and Baltic countries. It is also worth to note significant difference in the frequencies of the second by prevalence mutation p.R261Q between the two regions: 8.23% in Novosibirsk region versus 4.1 % in Kemerovo region.

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Table 1: PKU-associated *PAH* gene alleles frequencies

Location	Mutation			Allele numbers and frequencies	
	protein	cDNA	type	Novosibirsk region	Kemerovo region
exon 1	S16>XfsX1	c.47_48delCT	Deletion	1 (0.62%)	-
intron 1	IVS1+5G>T	c.60+5G>T	Splice	1 (0.62%)	-
exon 2	p.L48S	c.143T>C	Missense	2 (1.23%)	1 (1.4%)
intron 2	IVS2+1delG	c.169delG	Deletion	1 (0.62%)	-
intron 2	IVS2+5G>A	c.168+5G>A	Splice	1 (0.62%)	-
intron 2	IVS2+5G>C	c.168+5G>C	Splice	1 (0.62%)	-
intron 2	IVS2-13T>G	c.169-13T>G	Splice	1 (0.62%)	-
exon 3	p.R68S	c.204A>T	Missense	-	1 (1.4%)

intron 4	IVS4+5G>T	c.441+5G>T	Splice	3 (1.85%)	1 (1.4%)
exon 5	p.R155H	c.464G>A	Missense	-	2 (2.7%)
exon 5	p.R158Q	c.473G>A	Missense	7 (4.32%)	2 (2.7%)
exon 5	p.Y168H	c.502T>C	Missense	-	1 (1.4%)
exon 6	E221_D222>Efs	c.663_664delAG	Deletion	-	1 (1.4%)
exon 6	D222>STOP	c.664-665delGA	Nonsense	1 (0.62%)	-
exon 7	p.R243Q	c.728G>A	Missense	-	2 (2.7%)
exon 7	p.R243X	c.727C>T	Missense	1 (0.62%)	1 (1.4%)
exon 7	p.R252W	c.754C>T	Missense	1 (0.62%)	1 (1.4%)
exon 7	p.R261Q	c.782G>A	Missense	13 (8.02%)	3 (4.1%)
exon 7	p.R261X	c.781C>T	Missense	2 (1.23%)	-
exon 7	p.E280K	c.838G>A	Missense	1 (0.62%)	-
exon 7	p.P281L	c.842C>T	Missense	4 (2.47%)	2 (2.7%)
exon 10	p.A342T	c.1024G>A	Missense	-	1 (1.4%)
intron10	IVS10-11G>A	c.1066-11G>A	Splice	4 (2.47%)	3 (4.1%)
intron11	p.S349P	c.1045T>C	Splice	1 (0.62%)	-
exon 11	p.Y386C	c.1157A>G	Missense	-	2 (2.7%)
exon 11	p.E390G	c.1169A>G	Missense	1 (0.62%)	-
intron11	IVS11+1G>C	c.1199+1G>A	Splice	-	1 (1.4%)
exon 12	p.A403V	c.1208C>T	Missense	1 (0.62%)	-
exon 12	p.P407L	c.1220C>T	Missense	1 (0.62%)	-
exon 12	p.R408Q	c.1223G>A	Missense	1 (0.62%)	-
exon 12	p.R408W	c.1222C>T	Missense	103(63.58%)	41(56.2%)
exon 12	p.Y414C	c.1241A>G	Missense	2 (1.23%)	3 (4.1%)
intron12	IVS12+1G>A	c.1315+1G>A	Splice	3 (1.85%)	1 (1.4%)
	X			4 (2.47%)	3 (4.1%)

X- mutations in any exon or adjacent intron regions not found.

Table 2: PKU-associated *PAH* gene genotypes frequencies

Genotype	Novosibirsk region	Kemerovo region
p.R408W/p.R408W	30 (38.5%)	13 (38.2%)
p.R408W/p.R261Q	9 (11.5%)	1 (2.9%)
p.R408W/p.R158Q	6 (7.7%)	1 (2.9%)
p.R408W/IVS10-11G>A	3 (3.8%)	1 (2.9%)
p.R408W/IVS4+5G>T	3 (3.8%)	1 (2.9%)
p.R408W/p.P281L	2 (2.6%)	2 (5.9%)
p.R408W/p.R261X	2 (2.6%)	1 (2.9%)
p.R408W/p.Y414C	1 (1.3%)	2 (5.9%)
p.R408W/p.E390G	1 (1.3%)	2 (5.9%)
p.R408W/IVS1+5G>T	1 (1.3%)	1 (2.9%)
p.R408W/p.L48S	1 (1.3%)	1 (2.9%)
p.R158Q/p.R261Q	1 (1.3%)	1 (2.9%)
p.L48S/p.A403V	1 (1.3%)	1 (2.9%)
p.R408W/IVS12+1G>A	3 (3.8%)	
p.R408W/X	2 (2.6%)	
p.R408W/S16>XfsX1	1 (1.3%)	
p.R408W/IVS2-13T>G	1 (1.3%)	
p.R408W/IVS2+5G>A	1 (1.3%)	
p.R408W/p.R68S		1 (2.9%)
p.R408W/p.Y168H		1 (2.9%)
p.R408W/E221_D222>Efs		1 (2.9%)
p.R408W/D222>STOP	1 (1.3%)	
p.R408W/IVS2+5G>C	1 (1.3%)	
p.L48S/p.R158Q		1 (2.9%)

p.R408W/p.E280K	1 (1.3%)	
p.R408W/p.S349P	1 (1.3%)	1 (2.9%)
p.R408W/p.Y386C		1 (2.9%)
p.R408W/p.P407L	1 (1.3%)	1 (2.9%)
p.R408W/p.R408Q	1 (1.3%)	
IVS2+1delG/p.P281L	1 (1.3%)	
p.R155H/p.R155H		1 (2.9%)
p.R408W/p.R243X		1 (2.9%)
p.R243Q/p.R243Q		2 (5.9%)
p.R243X/X	1 (1.3%)	
p.R252W/p.Y414C		1 (2.9%)
p.R261Q/p.P281L	1 (1.3%)	
p.R261Q/IVS10-11G>A	1 (1.3%)	1 (2.9%)
p.R261Q/IVS12+1G>A		1 (2.9%)
p.R261Q/X	1 (1.3%)	
p.P281L/p.A342T		1 (2.9%)
IVS10-11G>A/IVS11+1G>C		1 (2.9%)
p.Y386C/X		1 (2.9%)

X- mutations in any exon or adjacent intron regions not found

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Peculiarities of Blood Group Distribution among Infants Born to Mothers with Negative Rh-Factor (Findings of 2014)

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Abstract

Our works consider the investigation of possible manifestation of hyperbilirubinemia in infants against the ground of genetic incompatibilities of the fetus according to ABO system and Rh-factor (D) concerning the maternal organism. From this point of view we deal with jaundice of mixed genesis against erythroblastosis domination as a primary antenatal factor of pathological process formation.

The present study presents the results of distribution of the group and rhesus determinants (Rh D) of infants born to mothers with negative Rh-factor in 2014. Analytical review and comparison with the previous investigations in this direction have been made. New trends of further work with the elements of chronobiological characteristics concerning possible signs of hemolytic diseases of newborns (HDN), neonatal isoerythrolysis, in Bukovyna region are outlined. The values of umbilical bilirubin concentration are taken as a biochemical criterion of HDN development which is the main diagnostic sign of pathological jaundice of newborns. The signs of HDN of various degree were observed in 24 infants out of 333 neonates born to mothers with negative Rh-factor during the period of 2014.

Keywords: hemolytic diseases of newborns (HDN), negative Rh-factor (anti-D), jaundice form, bilirubin concentration, Bukovyna region.

Introduction

Neonatal hyperbilirubinemia for the last decade has been characterized by the tendency to increase its possible signs during the neonatal period which is first of all explained by the spread of intrauterine infections, indirect influence of numerous lesions of the uterine-placenta complex during the antenatal period. All these factors together with the action of many medical agents form hepatobiliary insufficiency of neonates characterized by lees or higher degree of manifestation [1, 2].

Our works consider the investigation of possible manifetsation of hyperbilirubinemia in infants against the ground of genetic incompatibilities of the fetus according to ABO system and Rh-factor (D) concerning the maternal organism [3-6].

From this point of view we deal with jaundice of mixed genesis against erythroblastosis domination as a primary antenatal factor of pathological process formation. Fetal erythroblastosis

by AB0 system is known to constitute ⅔ of all the cases and ⅓ of cases – is incompatibility by the system of Rh-factor. It is interesting to know that erythroblastosis develops not in all the cases of incompatibility of the mother-infant system [7].

The level of umbilical bilirubin within the limits of 51,3-68,4 micromole/L is indicative of possible development of jaundice with indirect hyperbilirubinemia (hemolytic diseases of newborns, polycythemia, acquired and congenital hemoglobinpathy and enzymopathy) which is one of the causes of pathologic hyperbilirubinemia. The value of umbilical bilirubin concentration within the limits of 85,5-153,9 micromole/L is indicative of the development of jaundice with direct hyperbilirubinemia, that is, with domination of bilirubin-diglucuronide in the blood serum [7].

It should be emphasized that in infants with pathologic jaundice, only by clinical-anamnestic findings without laboratory tests initiated, the correct diagnosis is made only in 15% of all the cases.

As a rule, fetal erythroblastosis on the second-third day of life of an infant is characterized by adjoining of metabolic hyperbilirubinemia due to initial adaptation to new conditions of the surroundings.

Pregnancy with possible transplacental transmission of antibody-producing cells from mother to fetus is an important way of alloimmunization of the population especially in the areas of gene penetration including Bukovyna as well. Sensitization index calculated by the ratio containing the number of individuals with antibodies against general number and expressed in percentage will be considerably high for maternity homes and essential for local application in the medical establishment as a certain prognostic criterion. It will not be usually a reliable criterion for the region on the whole, but it will enable to prognosticate possible signs in mother-infant system and will be important to diagnose its spread among the population [8, 9].

During several recent years on the base of the Maternity Home № 1 in Chernivtsi, Ukraine, the distribution of groups (by AB0 system) and Rh-factor has been examined in the blood of infants born to mothers with different group determinants according to AB0 system, the value of umbilical bilirubin concentration was used as a prognostic criterion of hemolytic disease of newborns.

The article presents the analysis of the findings concerning the distribution of the criteria chosen among infants born to mothers with negative Rh(-) factor irrespective of the blood group. The results obtained were compared with the findings of 2013 (from January including July) with the aim to compile possible chronobiological characteristics of group distribution with the peak (maximal and minimal) values of hyperbilirubinemia in future.

Materials and methods

Laboratory examinations of the umbilical and maternal (in case of necessity) blood were made in the laboratory at the Department of Anaesthesiology with beds for intensive care units at the Maternity Home № 1, the town of Chernivtsi.

Detection of the blood group and Rh-factor was conducted according to the Order №164, the Ministry of Public Health of Ukraine dated 05.07.1999 “On Approval of the Instructions Regulating the Work of Blood Banking Establishments in Ukraine”, and “The Instruction to Detect Blood Groups and Rhesus by AB0 Systems” in particular [10].

Belonging to the blood group of patients was examined by means of agglutination reaction with the following reagents: standard serums and standard erythrocytes and monoclonal antibodies (coliclones anti-A, anti-B and anti-AB).

Standard serums and erythrocytes to detect blood groups were prepared in the laboratory at the Blood Banking Establishment of Chernivtsi Regional Center of Blood Banking (CRCBB). All the reagents were marked with the series and expiry date. Standard erythrocytes were prepared from the donor blood (according to the Instruction of taking and registration of blood received from donors in small doses to prepare standard erythrocytes). Monoclonal antibodies – erythrotest-coliclones produced in the Russian Federation, the company “Hematolog”, Moscow, certified on the territory of Ukraine were used in the study. Rh-factor was detected by anti-Rh_0(D) IgM monoclonal reagent produced by the company “Hematolog”.

The level of total bilirubin (TBR) and its fractions as one of the important biochemical prognostic criteria of HDN development was detected in the umbilical and infant blood.

Bilirubin and its fractions were detected according to the unified technique by Yendrashyk' method with the set of reagents produced by the company "Reagent" (Dnepropetrovsk, Ukraine). Photoelectrocolorimeter KFK-2 was used as a device to measure optic density of solutions.

The results were processed statistically with the detection of arithmetical mean value M and its error m ($\sigma\sqrt{n}$). Rank Kraskal-Wallis criterion was used for the comparative analysis of sampling.

Results and discussion

During 2014 on the base of the Municipal Clinical Maternity Home №1, Chernivtsi, 333 infants were born to 330 mothers with Rh(-) factor of blood by anti-D system through physiological labour of cesarean section. The majority of pregnancies were monocyesis, but twins were born: one to the mother with A(II) blood group and two to the mothers with AB(IV) blood group, mothers with B(III) blood group gave birth to stillborn twins, one was viable, that did not change general number of infants in this group concerning the number of women. It was interesting to know that the mothers with 0(I) blood group did not give birth to twins both in 2014 and in the first half of 2013. The majority of twins, six out from eight, inherited A(II) blood group as a dominant for our region. Out of general number of mothers with Rh(-) factor of blood the number of infants with A(II) blood group were 135: 75 from them were with positive Rh-factor. At the same time, during seven months of 2013 there were 83 newborns with A(II) blood group (64 with Rh(+) and 19 with Rh(-) factor) out of general number of newborns – 209 [11].

The distribution of group determinants of mothers and infants is presented in Table 1. The comparison of the distribution of blood groups both in 2014 and in the first part of 2013 revealed the priority of A(II) blood group both among mothers with negative Rh-factor and newborns, followed by 0(I), B(III) and AB(IV) respectively.

It should be noted that the majority of infants inherited maternal blood group. Only those born to mothers with AB(IV) blood group presented the distribution between A(II) and B(III) blood groups. In case to keep to the theory of development of AB(IV) blood group due to mixed marriages in the evolutional process but not under the influence of environmental factors, this kind of distribution appears to be quite natural and regular [12].

Mothers with AB(IV) Rh(-) blood group rarely give birth to babies with 0(I) blood group: one infant with 0(I) Rh(+) – during 2014 and none of the newborns with these characteristics during 2015. During the period of investigation in 2013 one newborn with 0(I) Rh(-) blood group born to the mother with AB(IV) Rh(-) blood group was characterized by a classical manifestation of HDN by ABO system: the level of umbilical bilirubin was 158,7 micromole/L, a substitute blood transfusion was performed twice while staying in the Department of Neonatology of the Maternity Home. The concentration of umbilical bilirubin was 158,7 mciromole/L, and as a statistical value it was the highest for the manifestation of all the types of erythroblastosis during the last five years in our Maternity Home.

Table 1: Distribution of group and rhesus determinants in infants born to mothers with Rh(-) factor of blood group during 2014

Blood group (ABO) and Rh –factor	0(I) infant		A(II) infant		B(III) infant		AB(IV) infant		$\mathbf{N}_{infant}$
	Rh(–)	Rh(+)	Rh(–)	Rh(+)	Rh(–)	Rh(+)	Rh(–)	Rh(+)	
0(I) Rh(–) mother (n=97)	**27**	37	11	9	6	7	---	---	97
A(II) Rh(–) mother (n=130)	10	13	**33**	50	5	10	5	5	131
B(III) Rh(–)	6	5	5	5	**8**	19	3	10	61

$_{mother}$ (n=61)									
AB(IV)Rh(–) $_{mother}$ (n=42)	---	1	11	11	10	3	**1**	7	44
N$_{infant}$	43	56	60	75	29	39	9	22	333

Notes: the number of maternal and infant groups do not coincide due to the birth of twins; the numbers concerning identical reproduction of maternal signs according to the characteristics examined in infants are written in boldface font

According to the scientific data AB(IV) blood group does not possess immunity to the antigens A and B. The Ukrainians inherited AB(IV) blood group due to historical-geographical migration of the Pechenihies, Hungarians and Polish Jews, its spread is proved to coincide with the areas of population settlement of the Jews and Gipsy [12].

The Ukrainian population is characterized by the prevailing A(II) blood group by AB0 system.

The concentration of total bilirubin in the umbilical blood was characterized by a wide range of values: from 25,3 to 124,2 micromole/L infants from all the groups irrespective of the maternal blood group. HDN manifestation was found in 24 cases out of general number of newborns (333 infants). It is interesting to note that the value of umbilical bilirubin in case of HDN manifestation was also characterized by a wide range of indices: from quite safe 29,9 to maximally critical 124,2 micromole/L. The concentration of umbilical bilirubin 124,2 micromole/L was a record in 2014.

The greatest amount of HDN manifestation – 12 cases, that is, 50% out of general number, was found from mothers with 0(I) blood group. To the point, a record value of umbilical bilirubin was found in an infant with 0(I) Rh(+), who was born to mother with 0(I) Rh(-) blood group: a classical incompatibility by the Rhesus-factor system. All other cases of HDN in this group of infants were different combinations of the blood group and Rhesus-factor except B(III) Rh(-) blood group of infants who did not present erythroblastosis signs.

According to the literary data the majority of HDN cases is found by AB0 system than those found in Rh(D) system in the ratio 3:1 [7, 13].

Thus, HDN is characterized by extremely variable values of the umbilical bilirubin concentration which is a valuable initial criterion of the development of pathological jaundice but not the only one.

Especially interesting manifestation of HDN there was a case with a mother with A(II) Rh(-) blood group given birth to an infant with identical blood group by AB0 system but positive Rh-factor by Rh(D) system. The umbilical bilirubin level was 78,2 micromole/L, which was quite real in this case of erythroblastosis. Although the value of direct bilirubin concentration as a fraction of the total one was 48,3 micromole/L, being an alarming symptom and drawing the attention of neonatologists. As in the majority of cases HDN is characterized by the total bilirubin value at the expense of the content of indirect fraction. Thrombocytopenia was diagnosed in this newborn. Primary forms of thrombocytopenia of alloimmune or isoimmune character can occur due to the transmission of platelets from the fetus to mother (as it happens during Rhesus incompatibility) or blood group incompatibility of platelet antigens in the pregnant-embryo system [14].

An average content of total bilirubin in the umbilical blood of neonates irrespective of the maternal blood group was within the same rates: 36-38 micromole/L (Table 2).

Table 2: An average level of total bilirubin (micromole/L) in the umbilical blood depending on the maternal blood group with Rh(-) factor (2014)

	0(I)	A(II)	B(III)	AB(IV)
M ± m	37,95 ± 1,19	36,87 ± 0,78	37,85 ± 1,37	36,87 ± 1,37

Notes: M – arithmetic mean of bilirubin concentration and its error m=(σ√n).

The data presented in the table are indicative of the similar average content of total bilirubin in the umbilical blood irrespective of the maternal blood group, that is, the conversion of physiological jaundice into pathological one and the level of HDN manifestation are in reality individual characteristics of every newborn.

By means of Kraskal-Wallis criterion a cross comparison of the total bilirubin level in infants with the same blood group born to mothers with different blood group characteristics by AB0 system was conducted (for example, infants with A(II) Rh(+) born to mothers with 0(I), A(II), B(III), AB(IV) blood groups etc.). Thus, a leading criterion to compare and find a reliable difference in the total bilirubin level was the blood group inherited by an infant. Table 3 presents the average level of umbilical bilirubin in infants with B(III) Rh(+) blood group.

Table 3: An average level of total bilirubin (micromole/L) in the umbilical blood of newborns with B(III) Rh(+) blood group born to mothers with Rh(-) factor by D system (2014)

	$0(I)_{mother}$	$A(II)_{mother}$	$B(III)_{mother}$	$AB(IV)_{mother}$
M ± m	40,74 ± 4,42	37,26± 2,84	38,05 ± 1,66	36,03 ± 2,02

Notes: there is no reliable difference found between the groups by bilirubin level.

Analogical comparative analysis was made between newborns with different blood group characteristics concerning the value of umbilical bilirubin concentration. There were no reliable differences between the selected groups found by means of Kraskal-Wallis criterion, that is, inherited the same blood group and Rh-factor from mothers or different group determinants do not influence upon the average value of umbilical bilirubin. Therefore, there were no reliable differences found according to the blood group characteristics of infants concerning the maternal blood group determinant.

It should be noted that the most prominent manifestation of HDN was found among infants born to mothers with 0(I) Rh(-) blood group: 12 cases constituted 12,4% out of general number of neonates in this subgroup (in other subgroups this percentage varied from 4,6 to 6,5%) (Fig.). In addition, the analysis of distribution of group determinants from mothers with 0(I) Rh(+) blood group during 2014 demonstrated reliable differences of an average bilirubin level among infants with 0(I) Rh(+) blood group only in its comparison with diametrically opposite groups: with A(II) Rh(–) ($p<0,05$) and B(III) Rh(–) ($p<0,01$) [6].

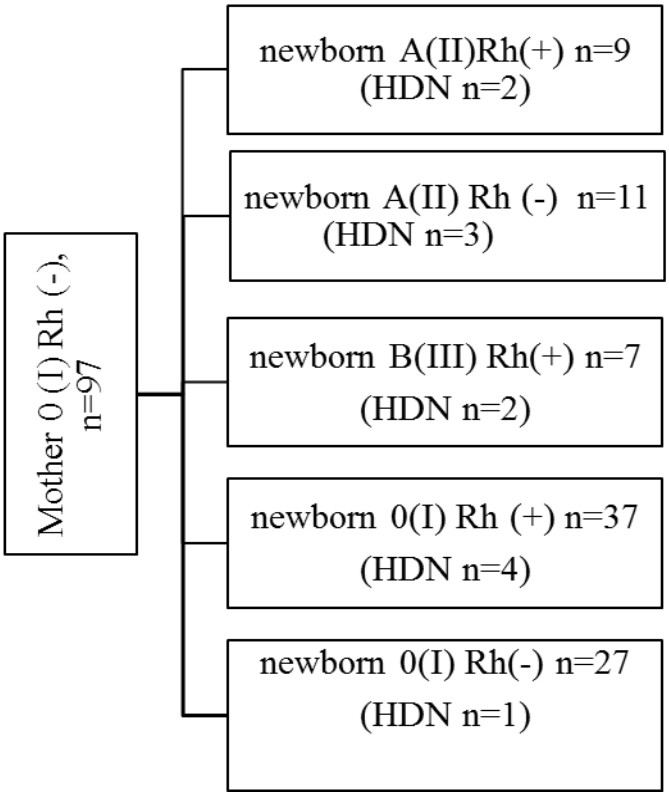

Figure 1. The number of HDN in infants born to mothers with 0(I) Rh(-) blood group by AB0 system during 2014

Hyperbilirubinemia is more often found in newborns with A(II) and B(III) blood groups born to mothers with 0(I) blood group, which is connected with anti-A and/or anti-B antibodies of IgG class present in their blood serum [15].

In case of double incompatibility both by the group and Rhesus-factor HDN caused by A- or B-antigens is likely to develop much easier than in case of isolated Rh-conflict. The birth of a baby with positive Rh-factor and incompatible blood group concerning the maternal one by AB0 system is likely to reduce the probability of immunization at the expense of competition for the antigen or "moderate" the course of jaundice

The family of Rh-proteins is known to be an important constituent of the erythrocyte cytoskeleton, they participate in the transportation of water and ammonium ions through the membrane. Rhesus-proteins Rh(D) are the molecules piercing the erythrocytic membrane 12 times in the direction from internal to external site, and again to the internal one; C- and N-ends of this protein are oriented into the cytoplasm site. Immunogenic characteristics of D antigen is not sufficiently studied [16, 17].

Conclusion

- umbilical bilirubin level is in fact an individual characteristics and not always an informative parameter to diagnose HDN;
- in early postnatal period it is difficult to diagnose HDN as it can develop in a latent form;
- bilirubinemia screening should be elaborated and introduced in the "risk group" of neonates (during the first 24-36 hours);
- Hb level is not always a prognostic criterion of HDN, first of all we deal with jaundice forms and transient polycythemia;
- umbilical bilirubin concentration within the limits of 50,0 micromole/L is the factor of a special attention for neonatologists (several years ago it was much higher – 65-70 micromole/L);
- possibility of fulminant development of HDN in a latent form and danger for further development of a newborn;
- increased frequency of HDN manifestation occurs with reduced light period (November-December-the first decade of January);
- various manifestations of HDN can be important modulators of the formation and further development of the immune system of infants.

These are the main issues for possible further directions of the investigation. In perspective a comparative analysis of the distribution of blood group characteristics in the mother-infant system will be made by the results of the studies conducted the previous year in our region including the characteristics of seasonal manifestation of HDN.

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HLA-G 14bp Deletion/Insertion Polymorphisms in Multiple Sclerosis

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Abstract

Background

The intensive researches during the last 40 years have found the human major histocompatibility complex (HLA) as the only locus conclusively associated with multiple sclerosis (MS). Recently, a possible influence of HLA-G in MS has been suggested due to its significant role in immune tolerance. One of them, HLA-G 14bp INS/DEL, has not been intensively studies and the published studies reported controversial results.

Aim

The aim of the present study was to examine the association of HLA-G 14bp INS/DEL and MS.

Methods

We present a case control study with 51 patients (familial MS 39%) and 51 healthy controls. All cases were with definite MS according to McDonald's criteria. The analysis of HLA-G 14bp INS/DEL was performed by PCR of DNA from peripheral blood.

Results

Overall comparison did not reveal statistically significant association between HLA-G 14bp INS/DEL and MS. A higher frequency of 14bpINS allele (60% vs 43%) and INS/INS genotype (40% vs 24%) was noted in the familial versus sporadic cases and controls. A significant correlation was found for genotype INS/INS in females. Surprising and reciprocal to the literature results were found in males. Genotype INS/DEL (high producers) was associated with higher risk, whereas INS/INS (low producers) was found to be protective.

Conclusion

HLA-G 14bp INS/INS is likely to be involved in familial MS and females in our population. The finding in males should be interpreted with a caution. The results warrant additional studies with international collaboration and larger sample size.

Keywords: multiple sclerosis, HLA-G 14bp INS/DEL.

Introduction

Multiple sclerosis (MS) is thought to be a result of complex interactions between unknown genetic and environmental factors. The intensive researches during the last 40 years have found the human major histocompatibility complex (HLA) as the only locus conclusively associated with MS (1). Although HLA I alleles - A*03 and B*07 were firstly reported, the strongest association has been found only with HLA-DRB1*15:01[1,2].

During the last decade, a possible role of HLA-G has been suggested due to its role in the regulation of the immune tolerance.[3-5] The role of different polymorphisms such as HLA-G 14bp INS/DEL has not been intensively studied and the preliminary studies reported controversial results. Therefore we sought to examine the role HLA-G 14bp INS/DEL and MS in a small pilot case-control study.

Material and methods

A case control study with 51 patients with definite MS diagnose by Mc Donald's criteria and 51 sex and age matched healthy controls was conducted. The disease course, long term disability and family history were assessed at the time of the sample collection. The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from all subjects prior to genetic testing.

DNA testing

DNA was extracted from peripheral blood. PCR primers was used to genotyped HLA-G 14 bp INS/DEL polymorphism (rs66554220).[6] Genomic DNA (100 ng) were amplified in a 25 μl reaction with a final concentration of reaction buffer (Genet Bio, Korea) 1×; each deoxynucleoside triphosphate 0.2 mmol/L; Taq polymerase (Genet Bio, Korea) 0.5 U and 0.5 μM for each primer. PCR amplification was performed with initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 61 °C for 45 s and 72 °C for 1 min and subsequently at 72 °C for 10 min. The products were visualized by electrophoresis on a 3% agarose gel (Applichem, Germany) containing ethidium bromide (0.5 μg/mL). PCR products were either 224 or 210bp, or both 224 and 210bp, according to the insertion/deletion of the 14bp in exon 8. The observed genotypes were directly counted by two different observers.

Statistics

Family history, clinical course and HLA-G 14 bp polymorphism were analyzed. We compared HLA-G 14bp allelic and genotype distribution between patients and healthy subjects, between sporadic and family cases and in subsets of patients with different disease course. Pearson's χ-test and odds ratios (ORs) were used for the purposes of statistical analysis, which was performed by SPSS, version 21.0 (Chicago, IL, USA). Additionally, the allelic and genotype frequencies of our Bulgarian healthy control subjects were compared with other populations.

Results

The mean age of the cases was 40.8 years (21-61), 35 women and 16 men. Familial MS was found in 20/51 (39.2%), 13 women and 7 men. In all of them there was maternal pattern of origin. Relapsing-remitting (RR) MS was found in 42 cases, relapsing-progressive (RP) in 5 and secondary progression (SP) after initial RR course was noted in 4. The mean age at the first symptoms was 30.4 (19-46) and the mean age of official diagnosis was 33.6 years (18-51). At the time of enrollment, the mean disease duration was 10.3 years (1-35) with a mean EDSS 3.68 (1.5-6.5). Past history of smoking and autoimmune diseases there was in 15/51 (29.4%) and 4/51 (7.8%), respectively.

The results are summarized in Tables 1-3.

Table 1: HLA-G allelic distribution

HLA-G	N	Aleles n (%)		p value	OR [CI]
		INS	DEL		
Controls	51	44 (43.1)	58 (56.9)	p>0.05	1.31 [0.75-2.28]
Cases	51	51 (50.0)	51 (50.0)		
Familial MS	20	24 (60.0)	16 (40.0)	p>0.05	0.51 [0.23-1.15]
Sporadic MS	31	27 (43.5)	35 (56.5)		

HLA-G – Human Leukocyte Antigen-G**, INS –** insertion**, DEL –** deletion**, OR –** Odds Ratio**, MS –** multiple sclerosis

Table 2: HLA-G genotypes distribution

HLA-G	N	Genotypes n (%)			p value	OR [CI]
		INS/INS	INS/DEL	DEL/DEL		
Controls	51	12 (23.6)	20 (39.2)	19 (37.2)	p>0.05	1.23 [0.50-3.00]
Cases	51	14 (27.4)	23 (45.2)	14 (27.4)		
Familial MS	20	8 (40.0)	8 (40.0)	4 (20.0)	p>0.05	0.4 [0.1-1.2]
Sporadic MS	31	6 (19.4)	15 (48.4)	10 (32.2)		

HLA-G – Human Leukocyte Antigen-G**, INS –** insertion**, DEL –** deletion**, OR –** Odds Ratio**, MS –** multiple sclerosis

Table 3: HLA-G genotype distribution by gender

		Case/control n (%)		p value (1-sided)	OR [CI]
	HLA-G	Cases	Controls		
males	**Ins/Ins**	0 (0)	6 (37.5)	**0.009**	**0 (0.000-0.0750)***
	Rest	16 (100)	10 (62.5)		
	N	16	16		
	Ins/Del	11 (68.8)	4 (25)	**0.016**	**6.600 (1.403-31.051)**
	Rest	5 (31.3)	12 (75)		
	N	16	16		
	Del/Del	5 (31.3)	6 (37.5)	0.500	0.758 (0.175-3.274)
	Rest	11 (68.8)	10 (62.5)		
	N	16	16		
females	**Ins/Ins**	14 (40)	6 (17.1)	**0.031**	**3.222 (1.063-9.768)**
	Rest	21 (60)	29 (82.9)		

N	35	35		
Ins/Del	12 (34.3)	16 (45.7)	0.232	0.620 (0.236-1.625)
Rest	23 (35.7)	19 (54.3)		
N	35	35		
Del/Del	9 (25.7)	13 (37.1)	0.220	0.586 (0.211-1.628)
Rest	26 (74.3)	22 (62.9)		
N	35	35		

*Statistically significant values are given in bold

Genotype distribution did not deviate from Hardy-Weinberg equilibrium among the healthy controls group. The comparison between cases and controls found no significant differences of the allelic and genotype distribution. When the patients group was divided according to the family history allele INS and genotype INS/INS were more frequent in familial than in sporadic cases and controls, but the differences were not significant.

Subsequently, the group of patients was divided according to the clinical characteristics to RR, RP and SP. Due to the small sample sizes (n≤5) of the groups of RP and SP patients, the p-values were not taken into consideration.

The sub-analysis by gender found significant difference between males and females (table 3). In males genotype INS/INS was found to be protective, whereas INS/DEL was associated with greater risk for MS. In females significant correlation with the risk for MS was found for the genotype INS/INS.

Discussion

HLA includes over 252 genes, located at 6p21 chromosome and grouped in two sub-regions – class I and class II. HLA I consists of two types molecules having different features – class Ia (classical - A, B, C) and class Ib (non-classical – E, F, G). In contrast to the classical class I (-A, -B and -C) molecules, HLA-G presents limited protein variability. Currently, 50 HLA-G alleles, encoding 16 functional proteins have been described and 2 null alleles.[7]

Seven HLA-G protein isoforms are result of alternative splicing of the primary transcript - four membrane-bound (HLA-G 1-4) and three soluble (HLA-G 5-7). HLA-G has unique structure, presenting a reduced cytoplasmic tail and capability to form dimers with enhanced LILR-mediated intracellular signaling.[8] It plays a significant role for establishing of the immune tolerance by Fas/FasL ligand mediated induction of apoptosis of CD 8^+ T cells and NK.[9,10]

Several single nucleotide polymorphisms (SNP) are described at DNA level, and those located in the promoter and 3'untranslated region (3'UTR) are frequently suggested to be functional. These polymorphic sites influence stability of mRNA and its turnover, mobility and splicing.[8,11] There are evidence that 14bp INS influences the mRNA stability and protein synthesis[12] and is also related to autoimmune diseases and some pregnancy-related conditions.[6,13] Two of them, 14bp DEL/INS and +3142C>G, have been proposed to be implicated in MS.[3] However they have not been studied intensively yet and there are few studies reporting conflicting results.

In series with 698 patients Kroner et al., reported no significant association with the susceptibility to MS, age of onset, severity or disease course in German population.[14]

A Polish case control study with 227 cases found significant correlation with three polymorphic sites of HLA-G.[15] On the background of similar distribution of 14bp INS/DEL in cases and controls the authors observed significant association with age at onset. The last was significantly higher in DEL/DEL homozygotes than in DEL/INS and INS/INS. Similar correlation they found for INS-positive vs. INS-negative cases.

In a recent series with 69 patients with relapsing-remitting MS, Rizzo and al., found that serum and cerebrospinal fluid levels (CSF) of soluble HLA-G are influenced by 14bp DEL/INS and +3142C>G polymorphisms.[4] The authors reported higher CSF levels of sHLA-G in MRI inactive than the cases with active disease. The combined analysis of 14bp INS/DEL and +3142C>G polymorphisms revealed the highest serum and CSF concentrations of sHLA-G in the high producers cases (DEL/DEL and +3142 C/C genotype) whereas the lowest titers were found in low producers (INS/INS and G/G genotype) both in active and inactive disease. This finding implicates

a possible role of HLA-G in MS on one hand, and that the release of sHLA-G in serum and CSF may be regulated not only by local environment but also by these two polymorphisms. There was no correlation of these polymorphisms with disease duration and disability.

Wiendl et al. assessed 11 brain specimens and cerebrospinal fluids of MS patients which compared to specimens from other neurological controls.[16] They reported significantly higher over-expression of HLA-G in the transition zone of chronic active plaques and in perilesional microglia cells than in healthy controls and overexpression of its receptor ILT-2 on macrophages and microglia. Moreover the authors observed stronger upreglation of HLA-G mRNA after Th1 than Th2 inflammatory stimulation. Another important finding represents the higher cerebrospinal fluid concentrations of soluble HLA-G in MS than in other neurological controls.

Fainardi et al., showed significantly higher cerebrospinal fluid levels of sHLA-G and lower levels of anti-apoptotic sFas in patients with RR-MS versus controls with other inflammatory and non-inflammatory neurological diseases in Italian population.[17] The analysis according to clinical and MRI activity showed increased levels of HLA-G and decreased Fas levels in MRI-inactive patients.

On one hand, sHLA-G may suppress the autoimmunity in MS acting as anti-inflammatory molecule. This effect is mediated by Fas/FasL-mediated apoptotic elimination of the activated $CD8^+$ T cells and natural killers. Additionally it shifted Th1/Th2 balance toward Th2 through the inhibition of $CD4^+$ Th1 cells proliferation and increase of IL-10 production.[5]

On other hand, HLA-G polymorphisms might determine the serum and CSF levels of sHLA-G irrespectively of the local inflammation. It is thought that the production of sHLA-G from HLA-G^{pos} T_{reg} is decreased in the patients with genotype INS/INS (low producers) and increased in INS/DEL and DEL/DEL genotypes (high producers).[18] This observation led to the hypothesis that the individuals with lower sHLA-G levels are at greater risk for MS than those with normal or higher levels, independent of the disease activity.

The present study found no significant discrepancy of allelic and genotype distribution between cases and controls. The frequency of INS allele INS/INS genotype in familial cases and sporadic cases were 60% vs. 44% and 40% vs. 19%, respectively. Similar result was observed when familial cases were compared with the controls (INS 60% vs 43%, INS/INS 40% vs. 24 %).

The analysis by gender revealed significant difference between males and females (table 3). A significant correlation was found for genotype INS/INS in females. Surprising and reciprocal to the literature results were found in males. Genotype INS/DEL (high producers) was associated with higher risk, whereas INS/INS (low producers) was found to be protective. In our opinion this finding should be interpreted with a caution.

An important drawback of our study is the small sample size due to financial limitations. In this light the lack association may reflect a type II error. This limitation might be overcome by multicenter collaboration in the future in which we may participate with the established MS-DNA bank.

Conclusion

Based on the results, the HLA-G 14bp INS/INS is likely to be involved in familial MS in our population and is associated with MS in females. The finding in males should be interpreted with a caution. The results warrant additional studies with international collaboration and larger sample size.

Conflict of interest: The authors have no conflict of interest.

Disclosure:

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Impact of Cantienica® Method to Improve Urinary Incontinence and Quality of Life

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Abstract

Background: The aim of this study was to determine the impact of symptoms of urinary incontinence by individuals, with the help of pelvic floor muscle's activation using Cantienica® and the subsequent effect on the quality of women's life.

Patients and methods: The selected sample consists of two groups - experimental and control. The experimental group consisted of 31 female patients with incontinence, who completed therapeutic exercises using features of Cantienica® method. Control group consisted of 31 female patients with incontinence, which had taken a different way of conservative therapy.

Results: The research has found that in the group of female patients who completed the therapeutic exercise by Benita Cantieni method, came in both phases to significantly greater change in the quality of life and greater alleviation of incontinence among the women who completed the other conservative treatments of incontinence.

Conclusion: It can be alleged by the observed results, that the evaluated method has an effect on improving the quality of life and alleviating the symptoms of urinary incontinence in women.

Keywords: Urinary incontinence. Pelvic floor. Body posture correction. Cantienica®. Quality of life.

Introduction

Urinary incontinence, despite the relatively frequently occurrence is still taboo. The negative impact of this problem is not only in health levels, but especially in the psycho-social and hygiene. Although there is no problem associated with high morbidity and mortality, it has a major impact on the quality of life of affected patients. It is not a disease in the true sense, but it is a symptom of the most different pathologies. Feedbacks from therapy, used treatments and their effectiveness are various. Only 35% of respondents expressed satisfaction with the quality of treatment (Švihra, 2009). Even in the assessment from some doctors we discovered the opinion that surgical treatment is far more successful than conservative treatment (Kawaciuk, 2009). Interdisciplinary approach also appears in other literary sources. Urinary incontinence is assessed as a serious economic, social and medical problem. So the diagnosis is necessary to distinguish the stress, urgent, mixed, or other types of incontinence. It helps anamnesis with using ICIQ-SF questionnaire, physical examination, including the tests for incontinence, residual urine measurement and laboratory testing of urine and blood. Examination by a specialist, Urologist, Urogynecologist, Neurologist is indicated, if there are presented complicating factors, when the basic examination does not help to determine the level and type of incontinence or in case of unsuccess of primary treatment of incontinence. In moderate stress incontinence is usually recommended regime measures and training of the pelvic floor, in urgent incontinence mostly antimuscarinic drugs and in other types the solution of the causes of incontinence (Lachváč, 2010). From the perspective of "popularity" of individual therapeutic action in patients dominates the collection devices. 93% of respondents have said that they use absorption facilities (Švihra, 2009). The results of therapy influences are also the way of access to the patient. It considers that the treatment of stress incontinence should always be strictly individual. The female patients have individual mobility problem, which cannot be generalized just because the consequence is the same and it is urinary incontinence. At the same time it is problematic individual control of the accuracy of activation of the pelvic floor muscles. There is missing Elementary control of correct implementation of exercises and the patient's motivation by a physical therapist. And exactly in this it sees the cause of laic opinion, but also the public expert, that physiotherapy incontinence does not bring the success. At the same time it expresses a negative approach to fairly widespread recommendation, that the "exercise" of the pelvic floor is being implemented by the system of interrupting the flow of urine during urination. After some time, it may in fact lead to damaging of proper voiding stereotype and then to the inability to completely empty your bladder (Krhut, 2005). Krhut (2005) assesses the physiotherapy as a full-featured therapeutic method in the treatment of incontinence. It has its indications and contraindications as such, especially cognitive limits. It can not be agreed with the opinion, that we indicate to the physiotherapy of those patients, who are unsuited for any other therapy. On the contrary, we consider physiotherapy in most cases as first-line therapy. The basic priority of physiotherapy in fact lies in the complete absence of side effects and in the case of therapeutic failure it does not exclude the use of any other method of treatment. Cantienica® method (Cantienica or method of Benita Cantieni) is anatomically correct, logical and complex. It provides guidance for the identification within the own body, it is an instruction for strengthening the muscles and for creating of significant basis not only for the successful treatment of incontinence, but also for its prevention (Cantieniová, 2007). Doctors, physiotherapists, midwives work abroad with this method. This method is suitable for all women and men of all ages category, non-sports people, and athletes. It consists of over 100 exercises, from simple to challenging. The individual exercises can be modified and adapted to the needs and physical condition of the patient. The therapeutic effect is usually seen after the first workout. The program of exercise is various and thereby is entertaining. Exercise is very pleasant for the body and gives it energy. The method is characterized by erect posture, the ideal position of the pelvis, ribcage, spine, leg bones, ensures optimal functioning of joints. The method is harmonious, develops a feeling for your own body, its holding and movement within everyday life, supplies a sense of lightness, harmonious movements, youthful radiation and forms the figure. The method is very practical, exercises can be used in everyday life (Cantienica, 2012). The object of research was urinary incontinence, women´s quality of life with stress urinary incontinence, the symptoms of urinary incontinence and accurately aimed exercises, which has used of the elements of Benita Cantieni method.

Patients and methods

The selected sample consists of female patients with incontinence the first and the second level, randomly divided into two groups - experimental and control. The experimental group consisted of 31 female patients with incontinence who completed therapeutic exercises using features of Cantienica® method. Control group consisted of 31 female patients with incontinence, which have taken a different way of conservative therapy. The only intentionally criterion for the selection of respondents was woman with urinary incontinence the first or the second level according to classification of Ingelmanna - Sundberg. The age of female patients was ranged from 37 to 69 years. The research was conducted from January 2014 to October 2014. The first phase of the research was accomplished for ten therapeutic units, where the duration of one therapeutic unit was of 60 minutes, periodically once a week. In the second phase of research the female patients adhered to complete program at domestic environment, where they had to repeat the exercise regularly several times a day for a further ten weeks. Throughout the research were conducted three surveys, input, controlling after 10 weeks and output after the next ten weeks, in all a total of 20 weeks. From the method of research we used two questionnaires - Incontinence Quality of Life Questionnaire I - QOL, International Consultation on Incontinence Questionnaire ICIQ - UI Short Form (Bushnell, 2005). After the completion of quantitative data's collection, the results of this research were statistically analysed using descriptive statistics and the tools of comparative analysis where we used non-parametric test types such as Mann-Whitney's test that is used to compare the median values of two independent samples. Its output is among other characteristics called p-value of the test. This p-value is compared with a level of significance α, which we have determined the most commonly used value of 0.05. And non-parametric sign test, which evaluates the number of changes that have occurred in the file and on the basis of it the p-value is calculated. Statistical calculations and their graphical representation were realized in the statistical program of Statistica 12.

Results

I-QOL – Incontinence Quality of Life questionnaire related to urinary incontinence and the problems associated with it. The questionnaire was completed by female patients three times in certain time intervals, followed by the total percentage evaluation the quality of life. Total score equals the sum of all items. The score is then transformed to a scale of 0 to 100% (Table 1).

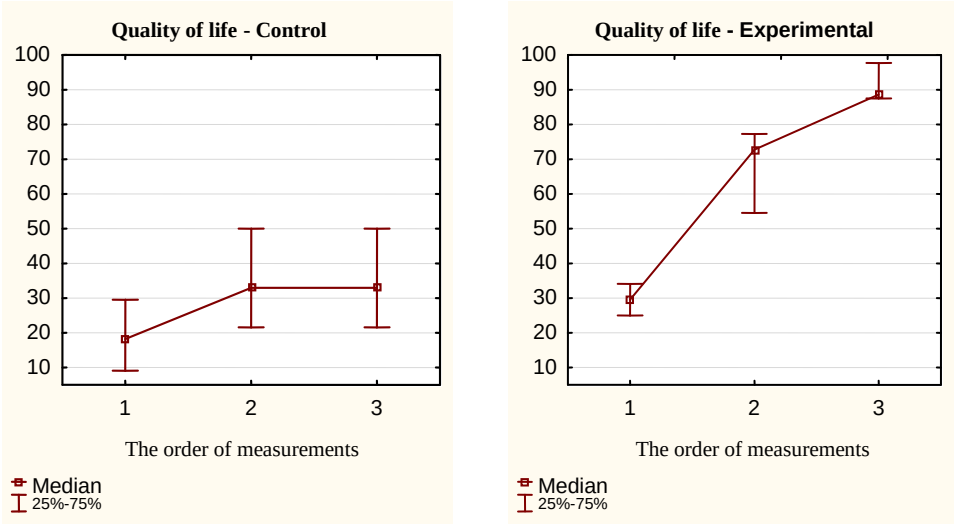
Table 1: Incontinence Quality of Life questionnaire – 3 measurements

Quality of Life I-QOL	***Control group***			***Experimental group***		
	1. measurement	2. measurement	3. measurement	1. measurement	2. measurement	3. measurement
Mean	***19,46***	***34,71***	***34,71***	***30,79***	***68,11***	***89,00***
Standard deviation	12,94	16,78	16,78	6,56	15,20	12,44
Coefficient of variation	*66%*	*48%*	*48%*	*21%*	*22%*	*14%*
Count	31	31	31	31	31	31
Minimum	0,0	9,1	9,1	20,5	25,0	50,0
Maximum	45,5	75,0	75,0	50,0	88,6	100,0
Variation margin	45,5	65,9	65,9	29,5	63,6	50,0

Lower quartile	9	22	22	25	55	88
Median	18	33	33	30	73	91
Upper quartile	30	50	50	34	77	100

During five months there was improvement in the quality of life in both control groups. In control group was the quality of life of 19.46 ± 12.94 at the beginning. The improvement was noted already at the second measurement, probably due to conservative treatment. Quality of life was at the second measuring 34.71 ± 16.78 points. Average of the group increased by more than 15 points. During the following 10 weeks the quality of life in this group remained unchanged. Even by the third measuring all female respondents evaluated it in average of 34.71 ± 16.78 points. It was found, that in the control group there was a slight improvement in the quality of life during the first phase. In the experimental group was greater significantly improvement of the quality. Already the steepness of the graph suggests at the same scale as a first graph, that the improvement is much higher in women who have completed special exercises (Graph 1).

The quality of life in this group was higher at the beginning then in the first group as it is shown above. At the beginning of the research the average was 30.79 ± 6.56. After the completing a series of exercises, which lasted for 10 weeks, the middle value of the quality improved by more than 28 points. In the second measurement there was an average of 68.11 ± 15.2. Gratifying is, that the improvement came also after the next phase, when the female patients had to take the exercise only at home. After another 10 weeks we have found by repeating request the quality of life in average 89 ± 12.44. This value is already very close to the maximum of 100 points and the improvement was in the second phase in the average of about 20 points.



Graph 1: Quality of Life – comparison of groups

It was assumed, that the experimental group of female patients will be achieving on average the higher output score than the control group. For verifying the obtained data was used the non-parametric Mann-Whitney test. For the comparing of two independent groups, it is the most frequently used of the parametric two-sample Student's t-test. However, the files do not fulfill the assumption of the test, therefore it was used as the non-parametric alternative. Table 2, 3 shows the basic characteristics of the calculated data and the characteristics of the test itself in the first and second phase of research.

Table 2: Changing the Quality of Life - 1st phase

Changing the Quality of Life - 1st phase Questionnaire I-QOL	**Control group**	**Experimental group**
Mean	15,25	37,32
Median	9,09	40,91
Mean rank	20,94	42,06
test statistic	Z = -4,606	
p-value	4,1E-06; (0,000)	

The output of the Mann-Whitney test is the average order of the values from the both compared files. The average order of data in the control group is significantly lower (20,94) than in the experimental group (40,91). The characteristics of the test: the testing statistic Z = -4.0606, and especially the calculated p-value -4,1-06, i.e. 0,000, testify for the significance of difference between the compared groups. P-value is smaller than usual level of significance of 0.05.

Table 3: Changing the Quality of Life - 2st phase

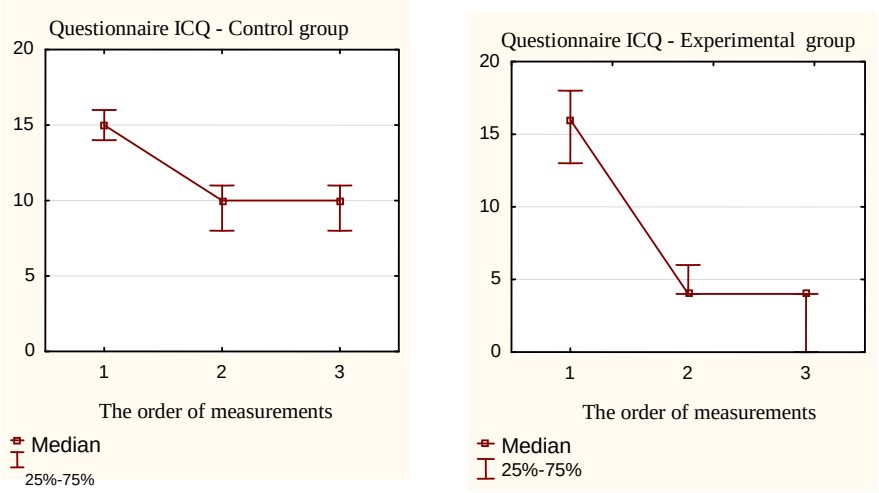
Changing the Quality of Life - 2st phase Questionnaire I-QOL	**Control group**	**Experimental group**
Mean	0,00	20,89
Median	0,00	18,18
Mean rank	16,50	46,50
Test statistic	Z = -7,043	
p-value	1,9E-12; (0,000)	

The output of the Mann-Whitney test is the average order of the values from the both compared files. The average order of data in the control group is significantly lower (16,50) than in the experimental group (46.50). The characteristics of the test: the testing statistic Z = 7.043 and especially the calculated p-value -1,9-12, i.e., 0,000, testify to the significance of differences between those two groups. P-value is smaller than usual level of significance of 0.05. It was found, that in the both phases has occurred in the group of female patients who have completed the therapeutic exercise by Benita Cantieni method to significantly greater change in quality of life than in the group of women who have completed other conservative treatments of incontinence. Questionnaire of the International Consultation on Incontinence ICIQ - UI SF assesses the symptoms of urinary incontinence. Scoring system of the questionnaire assesses the symptoms in the range from 0 up to 21 points. The female patients have received also this questionnaire overall three times at the same time as the questionnaire of quality of life. The processed results of measurements (Table 4).

Table 4: Questionnaire ICIQ – UI SF – 3 measurements

Questionnaire ***ICQ - SF***	***Control group***			***Experimental group***		
	1. measurement	2. measurement	3. measurement	1. measurement	2. measurement	3. measurement
Mean	15,16	9,42	9,52	15,16	5,13	3,32
Standard deviation	1,55	2,00	2,00	2,81	1,73	4,05
Count	31	31	31	31	31	31
Minimum	12	4	5	8	4	0
Maximum	18	13	15	18	10	16
Variation margin	6	9	10	10	6	16
Lower quartile	14	8	8	13	4	0
Median	15	10	10	16	4	4
Upper quartile	16	11	11	18	6	4

By comparing the average score's values of the questionnaire at individual measurement we find out a decrement of the score's values in both compared groups. In the control group was the average score before the research's beginning at the level of 15.16 ± 1.55 points. Already at the second measurement was recorded the reduced occurrence of symptoms of incontinence, as the average score dropped to a level of 9.42 ± 2.00 points. This score remained unchanged for the next 10 weeks and on the third measurement was the score on about the same level: 9.52 ± 2.00 points. In the experimental group was the decrement of the average score's values more significantly just after the first phase of research. At the beginning of this research was the average scores of the symptoms of incontinence in this group comparable to average of the control group. The value of the average is the same: 15, 16, but the variation is slightly higher. In this selection it reaches a value of 2.81. Already the second measurement finds the values of the score in experimental group in average about 10 points lower. The average score of the questionnaire after the first phase is 5.13 ± 1.73. After the second phase, in which the female respondents had to take a therapeutic exercise were the results of the score even slightly improved. The average value in third measurement was 3.32 ± 4.05. Notable is high the standard deviation in comparison to the acquired average. The high variability explains the maximum value - 16 points. The maximum is at the third measuring even higher than at the second measuring, where it reached a value of 10 points. As the lower quartile is as a minimum equal to zero, at least 25% of responding women have received at the third questionnaire of the symptom's scores of 0 points (Graph 2).



Graph 2: Symptoms of incontinence – comparison of groups

It was assumed, that the experimental group of female patients will achieve the average higher output score than the control group. For verifying the obtained data was used the non-parametric Mann-Whitney test (Table 5, 6).

Table 5: Symptoms of incontinence – 1st phase

Symptoms of incontinence – 1st phase	**Control group**	**Experimental group**
Mean	-5,74	-10,03
Median	-5,00	-10,00
Mean rank	43,29	19,71
Test statistic	Z = 5,168	
p-value	2,4E-07; (0, 00000)	

Table 6: Symptoms of incontinence – 2st phase

Symptoms of incontinence – 2st phase	**Control group**	**Experimental group**
Mean	0,10	-1,81
Median	0,00	-2,00
Mean rank	41,11	21,89
Test statistic	Z = 4,659	
p- value	3,2E-06; (0,000)	

The symptoms of incontinence did not change in general during the phases in the control group. The difference of ICIQ questionnaire is in the average between the second and the third measuring in an average of 0.10, but the median of change is equal to zero. However, in the experimental group, we have found a decrease of the score of incontinence. At an average, there was a decline of value of the group by 1.81 points. Because of the testing statistic is above the critical value and p-value of the test of 3.2E-06 is below the significance level of 0.05. It was found, that in both phases happened in the group of female patients, who completed therapeutic exercise by Benita Cantieni method, significantly to the greater alleviating of incontinence than in the group of women who have completed other conservative treatments of incontinence.

Discussion

The object of research was urinary incontinence, women's quality of life with the stress urinary incontinence, the symptoms of urinary incontinence and carefully targeted exercises, that it used the elements of the Benita Cantieni method. In the world, 65 million women suffer from urinary incontinence, 240,000 women in Slovak Republic suffer from urinary incontinence. About half of all women have this problem during their lives. Less than 5% of suffering patients from urinary incontinence look for a doctor immediately after the first symptoms, 60% of women begin to look for cure after a significant worsening of incontinence. The reason is that the incontinence still remains a taboo disease. More often it occurs in socially poorer groups, in women with the inferiority of tissues, that they suffer from varicose veins, hernias and all that. The urinary incontinence is the most common problem around the 45th year of life and in the elderly, which is probably related to hormonal changes during menopause and involution processes, caused by deficiency of estrogen. We meet with urinary incontinence also in women age of 23-35 years, although less frequently. We can say that the age limit of the incidents of incontinence is reducing (Vašínová, 2006). The adverse effects of urinary incontinence are severe; they include medical, psychological, economic and other problems. Medical problems represent irritation and skin maceration in the genital area and the possible origin of the infections. The psychosocial impact of urinary incontinence burden on the female patients and their families, the isolation increases, depression forms and the quality of life reduces. Urinary incontinence is an emotional burden; it carries with it a feeling of dirt, odors, which it often disrupts social and sexual relations. The economic consequences of the urinary incontinence are severe and the assumption is that by the ageing of the population they will still increase. The women with urinary incontinence are trying to fight with this problem alone for few years until they contact the gynecologist. If urinary incontinence is cured or at least improved, it would be a substantial improvement in the quality of life of individual patient (Džurný, 2002). The conservative treatment, behavioral, is less invasive than surgery, but its success depends on the interest and the cooperation of the female patient. The success of full recovery and improving of the condition is lesser than the surgical treatment. It is considered as a first line therapy. Conservative treatment includes the training of urine bladder, which is focused at the strengthening of pelvic floor muscles. Kegel exercises. Provides the control of urine leakage by the increasing of intra abdominal pressure. The effect of therapeutic gymnastic depends on the intensity and method of the exercise, whereby the main problem is the cooperation of the female patient. At the beginning of the treatment it is high, but it decreases over time (Mosnárová, 2003). The urinary incontinence largely affects everyday's life, reduces the quality of life, deteriorates the general health, but especially puts the patient into isolation absent from the social life and contacts. A patient is in many cases by the feelings of inferiority and shame enclosed to such an extent, that there will be disruption and collapse of the partner's relationship, and partner of the patient often does not know what is the real reason for their alienation. The urinary incontinence in this area becomes a significant psychosocial problem which if it is not solved, it means the patient losses self-esteem, limiting her daily activities and becoming enclosed from the community. As a consequence of this there is the loss of courage to confide their problems.

Conclusion

The urinary incontinence causes a significant deterioration in the quality of life. In many cases it is possible to completely cure it, or at least substantially alleviate the symptoms. Based on the findings of research it can be concluded, that the evaluated Cantienica® method has an impact on improving the quality of life and alleviate the symptoms of urinary incontinence. As it was demonstrated by the acquisition of a significant improvement in the group of women who completed the therapeutic exercises using the elements of Cantienica® method, and it in a greater change in the quality of life and a greater alleviation of incontinence as it happened in the group who has completed the other conservative treatments.

The human body, which has been created by nature, is beautiful. It is an integrated complex of the assorted organs and their functions, lead to the phenomenon - The life. During life the body is stimulated, encouraged, punished, rewarded and shaping by the world to which it was thrown. The body becomes something what it does and receives. And if it does not do, when it's done what we don't use and devastate, we will lose it (Farkašová, 2014).

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UDC 61

The Study of Some Anteroposterior Cranial Indicators on Cephalometric in a Vietnamese Group Age 18-25 with Normal Occlusion

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Abstract

Objective: assessement the sagittal relationship index on Cephalometric. **Subjects of study**: 42 Vietnamese aged 18-25 with normal occlusion. **Method:** clinical description on digital Cephalometric. **Results and conclusion**: SNA (male 82,64± 2,19; female 81,16±2,42); SN-Mp cắn (male 13,45±1,25; female 81,16±2,42); SNB (male 81,65± 2,25; female 79,85± 2,30); NPog – POr (male 85,84± 1,24; female 84,09±5,56); Angle Y(°) (male 61,71± 2,47; female 61,20±5,80), ANB (male 2,14±0,13; female 2,05±0,12); U1 – SN (°) (male 101,03±1,83; female 100,45±1,67); U1 –NA (°) (male 22,52±1,78; nữ 22,33± 1,88); U1–NA (male 5,10±0,61mm; female 4,99±1,28mm); U1-L1 (°) (male 123,38±2,27; female 120,36±2,05); L1 –MeGo (°)(male 94,98±0,80; female 94,48±0,82); L1 – NB (°)(male 94,98±0,80; female 94,48±0,82)

Keywords: cephalometric, vietnamese, normal occlusion.

I. Introduction

Vietnam is more and more developed and modern; people have increasing demand for life quality in general and aesthetic beauty in particular, especially facial aesthetics and smiles. Smiles with even teeth will make people become more attractive and confident, and also making a more beautiful face. 18-25 is the age when cranial development is almost complete [6], in addition Clo occlusion (according to Angle) is classified as normal occlusion or standard occlusion [1]. Therefore, it is necessary to survey and analyze indicators on cephalometric on subjects with normal occlusion to identify and propose norms of Vietnamese to serve orthodontics, aesthetic, anthropological identification for Vietnamese population. Due to such reasons, we have conducted this study with the objective: “Identifying indicators on cephalometric films in a Vietnamese group age 18-25 with normal occlusion”.

II. Subjects and research method

2.1. Research objects

* **Sample:** the research is conducted on 42 students of Hanoi Medical University age 18-25 with normal occlusion (21 male and 21 female).

* **Selection criteria:** Age 18-25. Have grown permanent teeth. Patients have enough 4 first molars and no milk teeth. Permanent first molars are not destroyed because of decay or they were decayed but have been filled. They have not had orthodontic treatment and other surgeries. They do not have diseases that affect dental, mandibular and facial development.

* **Elimination criteria:** not satisfying the above selection criteria.

2.2. Research methods: cross-section clinical description and on plaster sample.

Sample selection: random sample selection according to sample selection standard.

2.2.1 Clinical examination, getting bite marks and mould

- Devices: Examination devices: bean shaped tray, examination mirror, and dental picker. Bite mark substance: Alginate. Bite mark spoon. Stone plaster. Thin-leaf wax. Spirit lamp and alcohol 90^{o}. Rubber bowl, trowel to remove bite mark substance and plaster.

❖ Examine, take bite mark, pour mould, take occlusion wax in centric occlusion:

Take bite marks of mandible and maxilla teeth, form mould from stone plaster and get wax print in centric occlusion for all students meeting eligibility of the samples. Record information in research case history.

Criteria of plaster sample:

- Have from 28 to 32 teeth.
- Teeth in complete shape, unbroken and unchipped with no vesicle.

2.2.2 Identifying the type of occlusion on sample jaw:

Sample jaw is positioned in centric occlusion with occlusion wax. Use a soft black pencil to mark: axis of external knob near first molar of maxilla, external cavity near first molar of mandible. Depending on the relationship between external knob tip near big molar of maxilla and first big molar of mandible, we have different types of occlusion in jaw area according to Angle [1] as follows:

- Normal occlusion: external tip of first permanent big molar of maxilla fits external cavity of first permanent big molar of mandible. Teeth on jaw are arranged in an even occlusion line.

2.2.3 Cephalometric films for students with normal occlusion and film analysis

After classification of occlusion deviation according to Angle on sample jaws, students whose occlusion are identified to be normal on sample jaws will be taken Cephalometric at High-quality Odonto-Stomatology Centre (building A7) – School of Odonto-Stomatology – Ha Noi Medical University- VietNam. After that we begin the analysis of Cephalometric.

2.2.3.1 Devices

Examination device: Tray, dental mirror, dental pick, gương nha khoa, kẹp gắp, explorer brooch, gum measuring device, cotton-wool, gloves, illuminating lights and sterilizing devices. Digital cephalometric cameras: ORTHOPHOS XG. Computer. Software PLANMENCA ROMEXIS CEPALOMETRIC ANALYSIS 3.8.1.R.

Figure 1. Photograph showing Cephalometric Head Plate

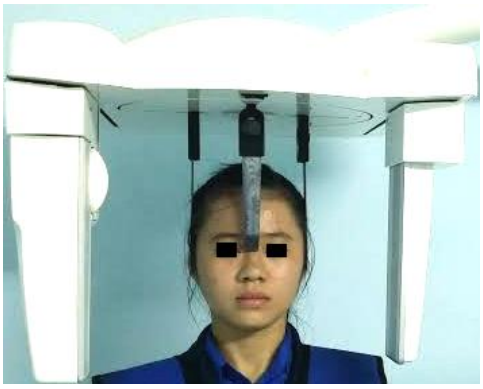

Figure 2. A female subject's head positioned in the Cephalostat. (Frontal View)

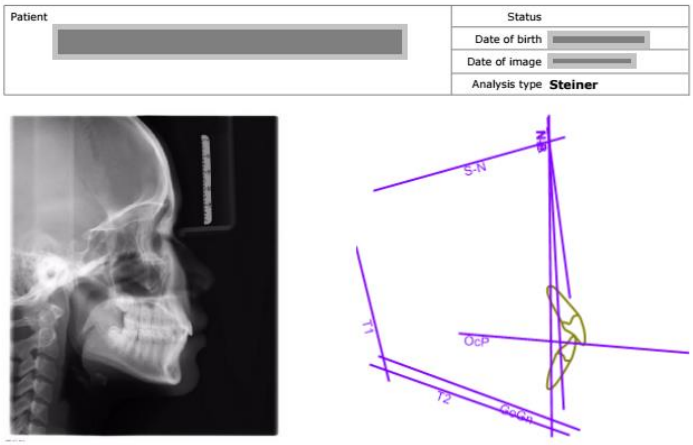

Figure 3. Results from software Planmenca Romexis Ceph. Analysis

2.2.3.2 Taking Cephalometric films for research objects

❖ Instruct objects about the correct position when taking a film.

❖ Criteria of Cephalometric films:

- General: can see clearly hard tissues and soft tissues.
- Specific: hard and soft landmarks of subjects' heads must be seen clearly. Including: external ear lob, lower part of eye socket, molars must be tight. Cranial base should reveal nasal bone, frontonatal suture. Maxillary reveals nasal cavity base, front nose spines, hollow bottom front nose, palate ceiling, butterfly slot, front teeth, the first large molars. Mandibular bone identifies chin protrusion, front, back, upper, lower part (both inside and outside) of the lower jaw, front teeth, the first large molars. Software identifies soft tissue circumference [7].

2.2.3.3 Film analysis

❖ Identify bone standard point:

- Midpoint nest hole: S. The nose: The banks of the lower orbital Nai: Or. Previous points nose spines: ANS. Postnasal barbed point: PNS. Most low points along the curve between XHT: A. The front teeth biting edge: Is. Point the tip of the upper incisors. Point edge incisor bite below: Ii. Point the tip of the lower incisors. The first big molars of upper jaw. The first big molars of lower jaw. Most low points XHD midline: B. chin Peaks: Pog. Chin point: Me. Most point and foremost under the chin: Gn. Mandibular angle point: Go. The highest point of the outer ear canal: Po
- Determine the planes straight lines under the horizontal plane: Plane (Mp) S - N: represent front cranial base, passing through the point S and Na. Palate plane: passing points ANS and PNS. Mp FH: passing points Or and Po. Bite plane: passing the midpoint of large molar teeth bite and the midpoint of the segment exhibiting the bite level of incisor. In case front teeth is open, bite plane passing through the midpoint of first molars bite and first small molar.
- Mandible plane: passing Me and Go.

- Identify angle, distances that need to be measured and measure such angle values và distances: SNA Angle: angle made of SN line cutting NA line at N point. SNB Angle: angle made of SN line cutting NB line at N. ANB angle = SNA – SNB. SN-OP Angle: made of SN line and bite plane. Facial angle (NPog – POr): angle made of line passing Na – Pog and FH plane. Y axis angle : acute angle made of S – Gn line and FH plane. Molar angle of maxilla and cranial base (U1-SN): angle made of SN line and line passing molar axis of upper jaw. U1 – NA Angle: angle between upper front teeth and N – A line. U1 – NA distance: distance from most prominent point of outer surface of molar to N – A line. U1-L1 Angle: angle made from line passing axis of upper front tooth and line passing axis of lower front tooth. L1-GoMe Angle: angle made of line passing axis of lower jaw front tooth and mandible plane (GoMe). L1-NB angle: angle made of line passing axis of lower front tooth and line passing Na – B. L1 – NB distance: distance from the most prominent point of front tooth of mandible to N–B line. Prominent angle of G-Sn-Pog':cute angle identified by two lines passing G – Sn and Sn – Pog' [2], [6].

❖ Record measurements in case history.

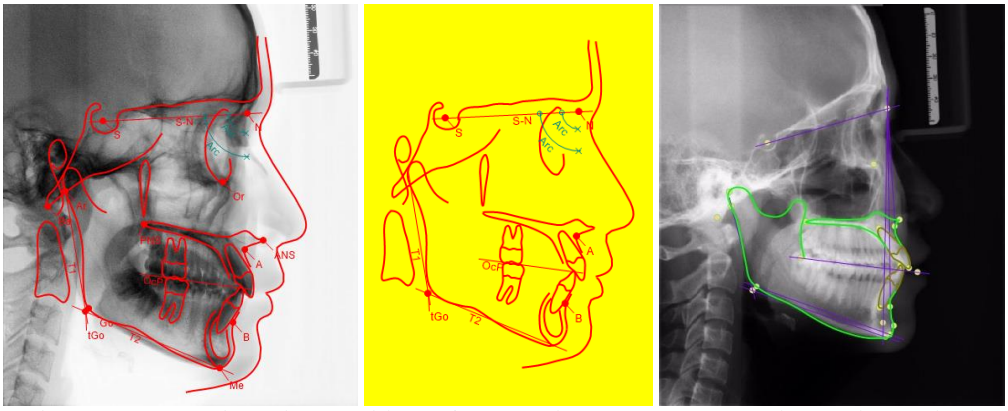

Figure 4: Landmarks used in software Planmenca Romexis Ceph.Analysis

2.2.4 Data cleaning and processing

- Clean data before analysis.
- Data is entered and analyzed by SPSS 20.0
- Verify variables by t- Test and χ^2

2.3 DEVIATION AND SOLUTIONS

2.3.1 Deviation

- ***Selecting research objects***

Errors in examining medical history and related diseases with odonto-stomatology.

- ***During taking cephalometric films:*** Technicians adjust parameters deviation, film mode, wrong position of ear – rode. Position of objects not meeting standards.
- ***During data analysis:*** Deviation during the identification of surgery landmarks.

2.3.2 Solutions

- Master theory of occlusion classification.
- Interview, clinically examine each object carefully.
- Select qualified and experienced technicians.
- Give specific instructions for subjects about head position, lip position during taking films. Practice many times before taking films.
- Practice to identify landmarks on films accurately.

2.4. Research period: from February 2015 to November 2015

2.5. Research ethics.

- The research is conducted at High-quality Odonto-Stomatology Centre of Odonto-Stomatology Training Institute - HMU.
- Explain to objects about research objective, responsibilities of researchers, responsibility and rights of participants.
- The study is only conducted on volunteers on the basis of co-operation, without obligation.
- All collected information serves the research objectives without any other purposes.
- During examination, if other dental diseases are discovered, patients will be consulted for

treatment or other examination methods will be conducted for accurate diagnosis.

- Research results will be sent to the School

III. Results

3.1. Gender distribution in research

Among the total of 42 objects, there is an equal proportion of male and female objects, each accounting for 50%.

3.2. Characteristics on cephalometric films of students with normal occlusion

3.2.1. Bone-bone corresponding indicator

Table 3.12: Bone indicators on Cephalometric (n=42)

	Gender	Male	Female	p*
	Indicator	$\overline{X}$ ± SD	$\overline{X}$ ± SD	
Upper	SNA (°)	82.64± 2.19	81.16±2.42	0.5066
	SN-bite plane (°)	13.45±1.25	13.64±0.91	0.5731
Lower	SNB (°)	81.65± 2.25	79.85± 2.30	0.2595
	NPog – POr (°)	85.84± 1.24	84.09±5.56	0.1653
	Y axis angle (°)	61.71± 2.47	61.20±5.80	0.7129
Upper – Lower	ANB (°)	2.14±0.13	2.05±0.12	0.9899

* ***t-test***

Remark:

There are no statistically difference about upper jaw bone, lower jaw bone indicators on Cephalometric film between male and female (p>0.05)

3.2.2.Bone-skeletal indicator

Table 3.13: Bone-skeletal indicator (n=42)

Gender	Male	Female	p*
Indicator	$\overline{X}$ ± SD	$\overline{X}$ ± SD	
U1 – SN (°)	101.03±1.83	100.45±1.67	0.6807
U1 –NA (°)	22.52±1.78	22.33± 1.88	0.7376
U1–NA distance (mm)	5.10±0.61	4.99±1.28	0.7350
U1-L1 (°)	123.38±2.27	120.36±2.05	**0.0001**
L1 –MeGo (°)	94.98±0.80	94.48±0.82	0.0508
L1 – NB (°)	23.89±1.58	30.70±1.43	**0.0000**
L1–NB distance (mm)	4.22±0.47	4.12±0.66	0.5780

* ***t-test***

Remark:

Average U1-L1 angle in male is 123.38±2.27 higher than female which is 120.36±2.05. This difference has statistical significance (p<0.05, t-test)

Average L1-NB angle in male is 23.89±1.58 lower than female which is 30.70±1.43. This difference is statistically significant (p<0.05, t-test)

Average U1-SN value of female is higher than male and in females there is a tendency of moving forward of front teeth compared with cranial base. This suggested that front molar is a little bit outward compared with cranial complexes in male.

There are no statistical significance in remaining indicators on Cephalometric films between male and female (p>0.05).

Iv. Comments:

From the analysis result of 42 Cephalometric films of students with normal occlusion with the 1:1 gender ratio (21 male and 21 female), we realize that:

Bone-bone relation: We have not found any evidence showing statistical difference in the value of SNA angle between the two genders in the study. Also when comparing with normal SNA angle (82°) there is no statistical significant difference. SNA indicator reflects the relative position of upper jaw bone compared with cranial base, the limit of this angle is 80-84°, if a big angle, upper jaw bone is moving forward compared with cranial base, if small angle, this proves that front jaw bones are less developed compared with cranial base [6]. Research findings show that this angle is 81.90° (male is 82.64° and female is 81.16°). Therefore, in both genders, SNA angle is still within normal limit compared with Steiner norm (82°), which means that upper jaw bone in relation with cranial base of Western people is no different from Vietnamese.

SNB angle reflects the relation between the middle position of lower jaw bone and cranial base, the bigger this angle is, chin and dental alveoli bone has more protrusion compared with cranial base. Permitted limit of this angle according to Steiner is 78°-82°. Our study also shows that, median values of SNB angle is 80.75° and of male 81.65± 2.25 (°), of female 79.85± 2.30 (°) and this deviation is not statistically significant. Therefore, SNB angle value of female is smaller than that of male.

ANB angle value describes the relation between upper jaw bone and lower jaw bone, with the cranial base as an intermediate. Our research finding: ANB angle value 2.09° in male is 2.14° and in female is 2.05°, this is suitable with research result of Steiner about normal value of ANB angle which is 2° [3], [4].

Skeletal-bone relation: our research shows, median values of U1 – SN angle (°) male is bigger than female, specifically: value of U1-SN angle in male 101.03±1.83(°) and in female 100.45±1.67(°). Similarly, U1 – NA angle value (°) male is bigger than female, specifically: U1-NA angle value of male is 22.52±1.78 (°) and in female are 22.33± 1.88 (°). Our research result is similar to normal U1-SN angle value published by Steiner which is 82° and normal value of U1-NA angle is 22° [3] [4].

V. Conclusion

Bone-bone relation: the relational position of upper jaw bone compared with cranial base SNA=81.90° (male is 82.64° and female is 81.16°). The relation between lower jaw bone with cranial base, this angle has median value of SNB=80.75° and in male is 81.65± 2.25 (°), in female is 79.85± 2.30 (°) and this deviation is not statistically significant. Therefore, SNB angle value in the research of female indicators is smaller than that of male. ANB angle value 2.09° in male is 2.14° and in female is 2.05°.

Bone-teeth relation: our research shows that median value of U1 – SN angle (°) male is bigger than female, specifically: value of U1-SN angle in male 101.03±1.83(°) and female 100.45±1.67(°). Similarly value of U1 – NA (°) angle in male is 22.52±1.78 (°) and in female is 22.33± 1.88 (°). The result is similar to normal value of U1-SN angle published by Steiner which is 82° and normal value of U1-NA angle is 22°.

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