

## **p. Leu72Met MUTATION OF GHRELIN *GHRL* GENE IN CHILDREN WITH GASTROESOPHAGEAL REFLUX DISEASE**

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### **Abstract**

Gastroesophageal disease (GERD) is the one of most spread diseases that injures an esophagus. Taking into account the large number of factors that can cause GERD development already in child age, including genetic predisposition, it is necessary to analyze each of them in detail.

Aim of this work was to analyze a frequency and possible association type of p. Leu72Met mutation of *GHRL* gene in children with gastroesophageal reflux disease.

The analysis of clinical parameters and course of disease in the group of 100 schoolchildren with GERD was carried out. The molecular-genetic study of c.214C>A locus of *GHRL* gene by CPR method (rs696217) was carried out in patients with GERD and 40 healthy children from the control group.

In 82 % of children with GERD was revealed a *GHRL* 214CC (Leu/Leu) homozygous genotype, at 58 % among children from the control group. *GHRL* 214CA (Leu/ Met) genotype was registered three times more seldom in children of the studied group comparing with ones from the control group: 15,0 % and 42,5 %, respectively. It was established, that *GHRL* 214CC genotype presence conditions the increase of GERD development risk in 3,4 times. On the contrary, *GHRL* 214CA genotype manifests a reliable protective effect that is essentially decreased GERD development risk in a child at such genotype – 0,24 comparing with a conventional unit.

There was established alleles and genotypes distribution of c.214C>A (p.Leu72Met) locus of *GHRL* gene in children with GERD. *GHRL* 214CC genotype is associated with threefold increase of GERD risk development. Gender differences as to GERD development risk at different genotypes of ghrelin gene were established. At *GHRL* 214CC (Leu/Leu) genotype the relative GERD risk for boys increased in more than 6 times. There were not established any differences in alleles and genotypes distribution depending on erosive or surface inflammatory changes of a gastrointestinal tract mucosa.

**Keywords:** gastroesophageal reflux disease, children, ghrelin, Leu72Met mutation.

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### **1. Introduction**

For today the one of cases of gastroesophageal reflux disease (GERD) is considered as a disorder of gastrointestinal tract upper parts motility with further emission of gastric acid in an

esophagus. The inhibition of stomach emptying is a risk factor of GERD development and is observed nearly in 10–40 % of patients (Festi and others, 2009) [1, 2]. The regular irritation of an esophagus mucosa by gastric acid causes inflammatory changes and in some cases erosions or ulcers. The distinct correlation was established between the duration of stomach content effect on an esophagus and severity degree of its mucosa injury [3]. It is well-known, that stimulation of gastrointestinal tract motility and appetite are greatly influenced by ghrelin (GHRL) [4, 5]. Ghrelin, orexigenic motilin-like hormone, discovered in 1996 by M. Kojima and his colleges, who reported about it in 1999 [6]. The most amount of ghrelin (over 90 %) is in a stomach and duodenum. Lower amounts were revealed in a placenta [7], hypophysis, arched kernel of hypothalamus [8]. Ghrelin can increase a speed of stomach emptying and so, decrease an amount of gastric acid that reaches an esophagus. Ghrelin anti-inflammatory effects were also presented in the studies by Golestan and others, 2010; Nikoukar and others, 2014. Thus, ghrelin has a positive influence on an esophagus mucosa through anti-inflammatory mechanisms [2]. Ghrelin secretion increases under conditions of the negative energetic balance such as hunger, cachexy and anorexia, whereas its expression decreases under conditions of the positive energetic balance such as feeding, hyperglycemia and obesity [9]. Ghrelin is expressed by GHRL gene, several SNP are informed for it. In literature is actively studied the SNP allele polymorphism with obesity [10, 11], food behavior disorders [12] and metabolism disturbances [13], and also with gastric acid secretion [14] and fast stomach emptying [15, 16]. Mutations in this gene can cause low levels of ghrelin, registered at obesity [17].

## 2. Aim of research

To analyze the frequency and possible association type of p. Leu72Met mutation of *GHRL* in children with gastroesophageal disease.

## 3. Materials and methods

The studied group consisted of 100 children 6–14 years old (in average 10,8 years), who underwent the stationary treatment at I-st pediatric department of the Municipal institution of Lviv regional council Lviv regional child clinical hospital (MI LRC LRCH “OHMADIT”) with a diagnosed pathology of gastrointestinal tract. The control group included 40 of the same age without any gastrointestinal tract pathology. Among the children of studied group were 44 boys (44±9,6 %) and 56 girls (56±9,6 %). All children underwent esophagogastroduodenoscopy using the videogastroscope “Fujinon” WG 88 FP, sonographic study of abdominal cavity organs, general clinical and laboratory studies, anamnestic data were analyzed. The group of 40 children without any manifestations of gastroesophageal reflux disease was formed as a control. The material of the study was 40 samples of DNA, separated from cells of bucal epithelium.

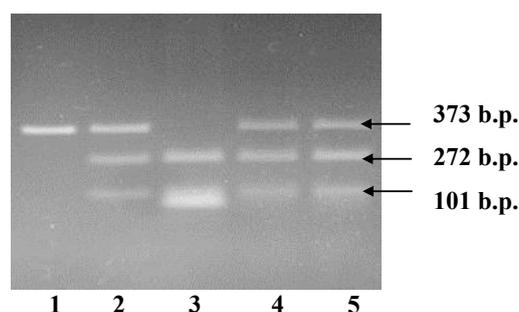
DNA of peripheral blood leucocytes was separated and purified by salting out method. PCR was carried out in automatic regime on the thermocycler “Tertsik” („DNA-technology, Russia), oligonucleotide primers, mixture of dDNTp and thermostable DNA-polymerase Thermo Scientific™ were used. The fragment of gene with the size 373 b. p. was amplified using primers (GREL2F 5'- TGA CCT CAC TGT TTC TGG AAG-', GREL2R 5'-GGA CCC TGT TCA CTG CCA C-3'). The analysis of PCR products was carried out by PRFL (polymorphism of restrictive fragments lengths) method. Amplification products were visualized by electrophoresis in 2 % agarose gel with ethidium bromide and scanned on the ultraviolet transilluminator “ECX-15. M (VILBER LOURMAT, France). The received signals were compared with lengths markers and sizes of received fragments were detected on this base. The results of agarose gel scanning were photographed by the digital camera “Gel Imager” (HELICON, Russia). The received data processing was carried out by conventional method of variational statistics with the calculation of odds ratio coefficient at 95 % credible interval.

## 4. Results

The anamnesis and course of gastrointestinal tract diseases were analyzed in 300 school-children. The criteria of inclusion in the studied group: complaints about gastrointestinal tract (pain in epigastral zone, nausea, heartburn, eructation, vomiting), apparent changes at the examination of

gastrointestinal tract organs using additional methods, voluntary consent of parents for genetic examination. The criteria of exclusion: refusal of the examination at any stage. The results, received at the endoscopic examination were interpreted using Savari-Miller classification in G. N. J. Tytgat et. al. modification, 1990. For setting the diagnosis “functional dyspepsia”, the Roman criteria IV were used. Among diseases of gastrointestinal tract in the studied group were revealed: erosive changes (erosive esophagitis, erosive gastritis, erosive duodenitis) – 16 children that is  $16\pm 7\%$  (4 girls and 11 boys); surface inflammatory changes (distal esophagitis, surface gastritis, surface duodenitis) – 84 children that is  $84\pm 7\%$  (48 girls and 32 boys). Gastroesophageal reflux disease with esophagitis was diagnosed most often among children of the studied group ( $59\pm 9\%$ ). On the second place by revelation frequency was a gastroesophageal reflux disease without esophagitis ( $26\pm 8\%$ ). Functional dyspepsia and a combination of duodenogastral and gastroesophageal reflux occurred in  $10\pm 6\%$  and in  $15\pm 7\%$ , respectively. There was not revealed any reliable difference in the frequency of gastrointestinal tract diseases depending on sex.

All children of the studied group underwent the molecular-genetic examination of alleles of Leu72Met locus of *GHRL* gene. As a result of PCR product processing by endonuclease of BsrI restriction, fragments with the following sizes were visualized on electrophoreogram: 373 b. p. – 214AA (Met/Met) genotype, 373 b. p. 272 b.p. and 101 b. p. – 214CA (Leu/Met) genotype, 272 b. p. and 101 b. p – 214CC (Leu/Leu) genotype, respectively. The electrophoreogram of molecular-genetic study of Leu72Met polymorphism of *GHRL* gene is presented on the **Fig. 1**.

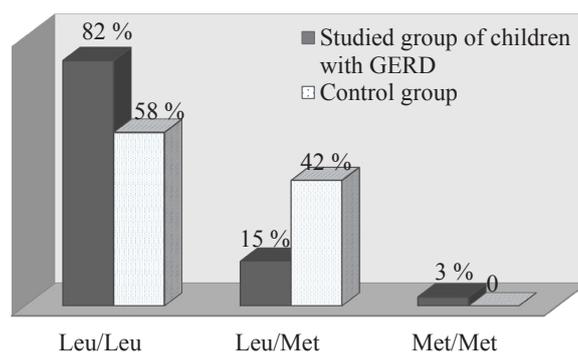


**Fig. 1.** Electrophoreogram of restrictive analysis of PCR products of SNP c.214C>A, p. Leu72Met polymorphism of *GHRL* gene 1 (2 % agarose gel): 1-Met/Met genotype (373 b. p.); 2,4,5-Leu/Met genotype (373 b. p., 272 b. p., 101 b. p.); 3-Leu/Leu genotype (272 b. p., 101 b. p.)

As a result of DNA molecular-genetic analysis in 100 persons of the studied group with GERD and 40 persons from the control group there was established a genotype as to p.Leu72Met polymorphic locus of *GHRL* gene. One-nucleotide polymorphism is conditioned by cytosine (214C allele) replacement by adenine (214A allele) and, correspondingly, amino acid replacement of leucine by methionine – Leu72Met. In 82 % of children with GERD was revealed *GHRL* 214CC (Leu/Leu) homozygous genotype, at 58 % among children of the control group. *GHRL* 214CA (Leu/Met) genotype was registered three times more seldom in children of the studied group comparing with ones from the control group: 15,0 % and 42,5 %, respectively. The results of genotypes distribution in children with GERD comparing with the control are presented on the **Fig. 2**.

As a result of the molecular-genetic analysis there were established statistically reliable differences in the ratio of *GHRL* gene genotypes between the group of children with GERD comparing with the control sample ( $\chi^2=12,94$ ,  $p=0,002$ ). The calculated coefficients of GERD odds ratio dependence on c.214C>A locus alleles of ghrelin gene are presented in the **Table 1**.

Taking into account the revealed differences as to p.Leu72Met polymorphic locus genotypes distribution of *GHRL* gene in children with GERD comparing with the control group, it was considered expedient to study allele frequency in the analyzed samples. Allele frequency indices of p. Leu72Met locus of *GHRL* gene in the studied groups of child patients are presented in the **Table 2**.



**Fig. 2.** Distribution of p.Leu72Met polymorphous locus genotypes of *GHRL* gene in children with GERD comparing with the control ( $\chi^2=12,94$ ,  $p=0,002$ )

**Table 1**

Distribution of p. Leu72Met polymorphous locus genotypes of *GHRL* gene in studied groups

<i>GHRL</i> genotype	Studied group, %	Control group, %	$\chi^2$	p	OR	
	n=100	n = 40			value	95 % CI
<i>GHRL</i> 214CC	82,0	57,5			3,37	1,50–7,56
<i>GHRL</i> 214CA	15,0	42,5	12,94	0,002*	0,24	0,10–0,55
<i>GHRL</i> 214AA	3,0	0,0			2,91	0,15–57,58

Note: n – number of persons, p – significance of differences in genotypes distribution between control and studied groups, \* – statistically reliable value, OR – odds ratio coefficient

**Table 2**

Allele frequency of p. Leu72Met polymorphous locus of *GHRL* gene in the studied groups

Alleles of <i>GHRL</i> gene	Frequency, %		$\chi^2$	p	OR	
	Studied group, % n = 100	Control group, % n = 40			value	95 % CI
<i>GHRL</i> 214C	89,5	78,8	5,63	0,02*	2,30	1,14–4,64
<i>GHRL</i> 214A	10,5	21,3			0,43	0,22–0,88

Note: n – number of persons, p – significance of differences between control and studied groups, \* – statistically reliable value, OR – odds ratio coefficient

As it is testified by the results, presented in the table 2, statistically reliable differences were established in the frequencies of normal C and low-functional A alleles of c.214C>A (p. Leu72Met) locus of *GHRL* gene in the studied groups of patients ( $\chi^2=5,63$ ,  $p<0,05$ ). The calculation of the odds ratio coefficient revealed a reliable effect as to A allele: OR=0,43, CI: 0,22–0,88 and association of C allele with the reliable increase of GERD risk in 2,3 times at the credible interval 1,14–4,64.

Gender differences as to alleles and genotypes distribution of c.Leu72Met locus of *GHRL* gene were analyzed (**Table 3**).

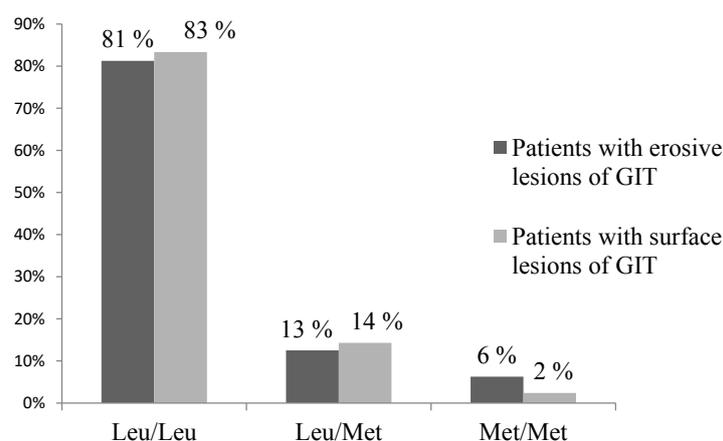
Thus, in 83,9 % of girls and 79,5 % of boys with GERD was revealed *GHRL* 214CC – Leu/Leu homozygous genotype that was similar to girls of the control group. The essential difference at gender analysis was observed among boys: the frequency of *GHRL* Leu/Met heterozygous genotype among boys of the studied group was 16 % and 60 % in the control ( $p=0,01$ ).

On the **Fig. 3** is presented the distribution in established genotype groups depending on injures severity (erosive changes and surface inflammatory changes) of a gastrointestinal tract.

**Table 3**

Genotypes distribution of c. Leu72Met polymorphous locus of *GHRL* gene depending on sex of patients of the studied groups

Genotype <i>GHRL</i>	Girls		Boys	
	Studied group, % (n=56)	Control group, % (n=21)	Studied group, % (n=44)	Control group, % (n=19)
214CC	83,9 (47)	76,2 (16)	79,5 (35)	36,8 (7)
214CA	14,3 (8)	23,8 (5)	15,9 (7)	63,2 (12)
214AA	1,8 (1)	–(0)	4,5 (2)	–(0)
	$\chi^2= 1,31, p=0,52$		$\chi^2= 6,54, p=0,01^*$	



**Fig. 3.** Distribution of p. Leu72Met polymorphous locus genotypes *GHRL* gene in children with GERD with erosive and surface inflammatory changes

There were not revealed any reliable differences as to the frequencies of certain genotypes of c.214C>A (p. Leu72Met) locus of *GHRL* gene in the studied groups of patients, distributed depending on gastrointestinal tract lesion severity.

## 5. Discussion

GERD occur in 8,7–17 % of children with gastroenterological diseases [18]. The world experience of treatment testifies that GERD early diagnostics decreases a risk of complications development. [2, 19] Extra-esophageal GERD manifestations [20–22], and revelation of typical esophageal symptoms only at 14–15 years old [23] conditions the necessity of searching new diagnostic possibilities. The presence of a genetic component in GERD development is proved by twin studies and provides multifactor etiology. [24] Thus, the hypothesis about predisposition genes of GERD development is reliable. Identification of such genes and alleles is topical for both early diagnostics and prophylaxis of GERD and for the further explanation of their development mechanisms through the comprehensive study of pathophysiological processes of these genes expression products. The connection between ghrelin and allele polymorphisms of its genes with digestive system diseases is specially discussed [2, 5].

In the work are presented the results of the distribution of alleles and genotypes of c.214C>A (p. Leu72Met) locus of *GHRL* gene in the group of schoolchildren with GERD comparing with the control group of children without a gastrointestinal tract pathology. As it is testified by the results of received data statistical processing and determined coefficient of odds ratio that represents a risk of GERD development, the differences in genotypes frequencies of the studied and control groups are significant like OR coefficients for *GHRL* 214CC and *GHRL* 214CA genotype (**Table 3**). It was established, that *GHRL* 214CC genotype conditions the increase of GERD risk in 3,4 times at the credible interval CI: 1,50–7,56. On the contrary, *GHRL* 214CA genotype demonstrates the credible

protective effect: OR=0,24, CI: 0,10–0,55. So, in a child with such genotype GERD development risk is essentially decreased – 0,24 comparing with a conventional unit. The high reliability degree of genotypes distribution differences was received at a premise of recessive type of 214C allele of *GHRL* manifestation ( $p=0,003$ ). The received data testify that 214C allele in homozygous state (Leu/Leu genotype) conditions the increase of GERD development risk in 3,4 times. There was shown the expressed protective effect as to *GHRL* 214A (72Met) allele of *GHRL* gene: the odds ratio coefficient is 0,43 at CI: 0,22–0,88 (**Table 2**).

It must be noted that ghrelin gene GERD risk allele is more spread in general population. The frequency of minor allele of *GHRL* 214A (Met72) in the control group was 21 %, and 11 % in the studied one. These results concern to Ukrainian population. It must be noted, that the study of such genetic locus among Ukrainians was not carried out, and we first inform about the frequencies of alleles of p.Leu72Met locus of *GHRL* gene. In different ethnic samples its frequency is 7,4–22,35 %. It was revealed, that the frequencies of Met72 allele in Europeans are 7,4–12,9 % (Vivenza and others, 2004; Bing and others, 2005; Magera and others, 2006; Zavarella and others, 2008) [25–28]; 18,1 % in Koreans ( Kim and others, 2006) [29] and ~20 % in Japanese (Ando and co-author, 2006; Kuzuya and others, 2006) [30,31]. The frequency of Met72 allele in our sample is 21 %, a bit higher than the data, given by HapMap (15,6 %, N = 90).

As it is testified by the data, presented in the **Table 3**, in this work were revealed certain features in p.Leu72Met locus genotypes distribution of *GHRL* in persons of the different sexes. Namely: absence of differences in girls group at essential differences among boys. Such results testify that the data about GERD risk at Leu/Leu genotype, received in the general studied group, are proved for boys with more reliability degree. Thus, the relative risk of GERD for boys at *GHRL* 214CC (Leu/Leu) genotype is increased in more than 6 times (OR=6,7, CI: 2,04–21,82). *GHRL* 214CA (Leu/Met) heterozygous genotype is protective as to GERD (OR=0,11, CI: 0,03–0,38) in boys and in general group.

## 6. Conclusions

1. The distribution of alleles and genotypes of c.214C>A (p. Leu72Met) locus of *GHRL* gene in children with GERD was established.
2. The genetic factor that increases GERD risk is 214 C allele of *GHRL* gene (Leu) in genotype. *GHRL* 214CC genotype is associated with a threefold increase of GERD development risk.
3. Gender differences as to GERD development risk at different genotypes of ghrelin gene were established. At *GHRL* 214CC (Leu/Leu) genotype the relative GERD risk for boys is increased in more than 6 times. *GHRL* 214CA (Leu/Met) heterozygous genotype is protective as to GERD boys and in general group.
4. Any differences in alleles and genotypes distribution depending on erosive or surface inflammatory changes of a gasrtointestinal tract mucosa were not established.

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