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Heat-shock protein 70-2 gene (1267A→G) in association with vulvovaginitis and cytokines levels in juvenile girls of Northern Bukovina

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Abstract
The aim of the study was to analyze the cytokines levels depending on 1267A→G polymorphism of HSP70-2 gene in 70 juvenile age girls of Northern Bukovina (Western Ukraine) with vulvovaginitis. The homozygous mutation of HSP70-2 gene (rs1061581) in study group was found in 2.86% cases, and was absent in control. Wild G allele of HSP70-2 gene present in every second teenager. The vulvovaginitis course in juvenile girls is accompanied by an increase of cellular immunity activity with prevailing of high and moderate production of pro-inflammatory cytokines (IL-1β and TNFα) 2.89 times (χ^2=33.03, p<0.001) (especially in the GG-genotype carriers by 12.13% (p=0.04) and 7.19 (p=0.055), respectively) with compensatory increase of humoral immunity activity by synthesis of anti-inflammatory IL-10 by 22.01% (p<0.001) and activation of local immunity of the vaginal mucosa by the secretory sIgA increase 2.6 times (p<0.001), which did not depend on allele condition of HSP70-2 gene. Genotypes and alleles of HSP70-2 gene (rs1061581) are not the additional risk factors of the vulvovaginitis in juvenile girls of Northern Bukovina.

Keywords: HSP70-2 gene (rs1061581), vulvovaginitis, juvenile girls.

1. Introduction
Heat-shock proteins (HSPs), in particular the HSP70 family, play important roles in intracellular trafficking, their conformation, removal of altered and denaturated proteins, acting as molecular chaperone, thus involving them in immune activity and regulation [1]. The HSP70 family includes constitutive proteins (HSP70c) essential for cellular function and the inducible form (HSP70i), which increases in response to environmental stress. Heat shock proteins adapt the immune system as they are carriers of the antigen peptides obtained from infected cells or tumors [2]. In addition, immunoregulatory role of HSP70 in the stimulation of cytokine production is proved [3], contributing to the immune supervision over an infected area or tumor [4]. Recently it was discovered that the HSP70i and HSP70c are encoded by highly related but differently regulated genes of the HSP70 multigenes family. There are three main genes of HSP70 family known, such as HSP70-1, HSP70-2 and HSP70-Hom, have been mapped to the class-III region of the human major histocompatibility complex. The genes HSP70-1 and -2 code practically similar inducible proteins which differ by two amino acids only, while HSP70-Hom codes non-thermal inducible protein, highly homological with the proteins of HSP70-1 and HSP70-2 genes [5]. Variations in the sequence of nucleotides of HSP70 genes influence upon the expression or function of HSP70 proteins, causing the changes of stress-resisting mechanisms, promote increased susceptibility to various pathological conditions including those associated with inflammatory diseases [6]. Variations of HSP70 gene are also related to the changes of oxidative stress activity in case of different pathology: in patients with diabetic foot [7], ischemic heart disease [8], hypertensive disease [9], pneumoconiosis [10], lymphoblast leukemia [11], cancer of the stomach and duodenal ulcer [12], idiopathic lung fibrosis [13] etc.
Nowadays there are no data concerning the participation of HSP70 genes in pathogenesis of inflammatory diseases of the minor pelvis in juvenile girls. Since polymorphisms of HSP70 genes play an important role in the immune system activity and can be one of the important causes of genetically determined dysregulation of inflammatory response, we considered it necessary to analyze A-1267G polymorphism of HSP70-2 gene (id.: rs1061581) in the structure of patients with vulvovaginitis among juvenile girls and define the probability of its influence upon the production of pro- and anti-inflammatory cytokines.

Consequently, the aim of our study was to analyze the cytokines levels depending on 1267A→G polymorphism of HSP70-2 gene (id.: rs1061581) in juvenile age girls of Northern Bukovina (Western Ukraine) with vulvovaginitis.

2. Materials and Methods

2.1 Compliance with bioethics

The study was performed in compliance with the Council of Europe Convention on Human Rights and Biomedicine and the Recommendations of the Committee on Bioethics of the Ministry of Public Health of Ukraine. Patients' Examination Cards and Patients' Informed Consent Forms were approved by the Biomedical Ethics Commission of Bukovinian State Medical University, the Ministry of Public Health of Ukraine (Chernivtsi, Ukraine). All enrolled patients were examined in the Department of Gynecology and Juvenile Gynecology of Maternity Clinical Hospital #2 (Chernivtsi, Ukraine) during 2013-2015 y.y. Genetic bench study performed in the laboratory of the Department of Medical Biology and Genetics of Bukovinian State Medical University. After screening (matching inclusion/exclusion criteria) 70 teenage girls were selected for further examination. The control group included 31 healthy teenage girls.

2.2 Inclusion / Exclusion criteria

Inclusion criteria. Juvenile age virgo girls (12-18 y.o.) with vulvovaginitis typical symptoms (primary acute vulvovaginitis or exacerbation of chronic vulvovaginitis) proved by clinical and gynecological examinations and depend on causes: grayish-white discharge (bacterial infection), genital itching and a thick, white vaginal discharge that is similar to cottage cheese (yeast infection), inflammation of the vulva and vagina due to parasites invasion, chemicals (vaginal contraceptives, feminine spray, perfumes, bubble bath, soap, etc.), sexually transmitted infections can cause genital discomfort, itching, and heavy discharge; nonspecific vulvovaginitis - mainly due to low estrogen, but usually has no known cause. No other gynecological disorders at the time of inclusion into study.

Exclusion criteria. We excluded patients younger than 12 y.o and older than 18 y.o.; presenting symptoms of other gynecological problems or extragenital inflammatory diseases; presenting undercurrent factors that can cause menstrual disorders (extra tube-ovarian and extra genital organ concomitant inflammation, pregnancy, anatomical genital anomalies, etc.); non-virgo pubescent girls who started sexual life; psychological disorders.

2.3 Diagnosis of vulvovaginitis

Selection of patients and their distribution into groups was performed according to the classifications of Ukrainian and International Societies of Obstetrics and Gynecology (International Societies of Obstetrics & Gynecology, European Society of Gynecology) [14-16]. The diagnosis of vulvovaginitis was made on the basis of the criteria of the acting national and international recommendations [14-16].

All patients were examined comprehensively: general clinical, gynecological, laboratory and instrumental examination (ultrasound of the pelvic organs).

2.4 Cytokines level investigation

The tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and IL-10 levels (in pg/ml) in blood plasma, and sIgA in vaginal secretion where detected by Immuno-enzyme method (ELISA) with the set of reagents of "Vector Best" (RU, ISO certificates 9001, 13485).

2.5 Genotyping of the HSP70-2 (1267A→G) gene polymorphism (rs1061581)

Genomic DNA was extracted from peripheral blood leukocytes using the "DNA-sorb-B" test system, with primers specific to the genes' alleles [17]. Detection of 1267A→G polymorphism of HSP70-2 gene was performed by the polymerase chain reaction (PCR) according to the manufacturer's protocol. Allele-specific primers were used in the PCR (Table 1). PCR amplification was carried out by 35 cycles of denaturing at 95 ℃ for 1 min, annealing at 54℃ for 30 s, and extension at 72℃ for 1 min. PCR products were then digested with PstI endonuclease restriction enzyme ("Fermentas", Lithuania) at 37 ℃ for 12 h. The HSP70-2 restriction fragment length polymorphism related to the polymorphic PstI site at position 1267 (restriction site - 5'…C T G C A↓G…3'; 3'…G↑A C G T C…5'): lacking the polymorphic PstI site generates a product of 1117 bp in size (allele A), whereas the HSP70-2 PstI polymorphism produces two fragments of 936 bp and 181 bp in size (allele G). The digested PCR products were separated by horizontal electrophoresis in a 2% agarose gel, stained with 4 μl of ethidium-bromide and visualized in the presence of molecular mass ladder (100-1000 bp) using a UV transilluminatior (Nyxtechnic, USA) (Fig. 1).

Table 1: Primer sequences for HSP70-2 (rs1061581) gene SNP and size of fragments

SNP locus	Primers	Primer sequences (5'-3')	Size of fragments, bp
Hsp70-2 (A-1267G) (GRCh38.p2)	Forward	5′- CATCGACTTCTACACGTCCA -3′	Allele A: 1117 bp
	Reverse	5′-CAAAGTCCTTGAGTCCCAAC-3′	Allele G: 936 bp, 181 bp

Note: bp – base pair

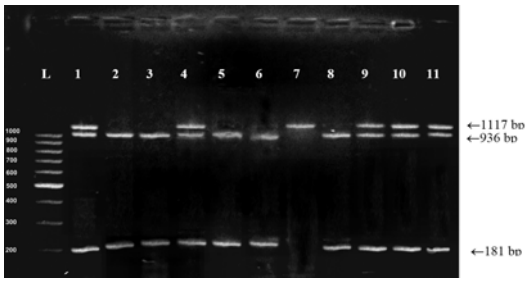

Fig 1: Electrophoregramm of human DNA PCR products amplification of Hsp70-2 A-1267G gene polymorphism. Note: L – DNA Ladder (1000-100 bp); lines 1, 4, 9-11 – heterozygous AG variant; line 7 – homozygous AA genotype; lines 2, 3, 5, 6, 8 – homozygous GG variant.

2.6 Statistical analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc, USA) software. P value and odds ratio (OR), with 95% confidence interval (CI) using a chi-square test were determined for the calculated frequencies of each allele and genotypes. Reliability of the data for independent quantitative sampling was calculated using Student's t-test (if distribution by Kolmogorov-Smirnov and W-Shapiro-Wilk test was close to the normal) and U-test *Wilcoxon-Mann-Whitney* (in case of irregular distribution). Risk ratios (RR) were estimated by OR. Adjusted OR and 95%CI were estimated for association between vulvovaginitis inflammation and genetic polymorphism. P values <0.05 were considered statistically significant.

Results and Discussions

70 juvenile girls with vulvovaginitis were screened. In the majority of the examined patients (64.29%) vulva and vagina inflammation was diagnosed for the first time in acute period, in others – exacerbation of chronic vulvovaginitis. The reference group included 30 healthy juvenile girls. The distribution of 1267 A→G genotypes of the polymorphism of HSP70-2 gene corresponded to the expected population balance of *Hardy-Weinberg*. The frequency "wild" G-allele of HSP70-2 gene in the juvenile girls of the study and control groups (Table 2) is 44,28% and 50,0% and that was larger than a "minor" A-allele (p<0.001). A relative frequency of G-allele and A-allele, as well as the genotypes of HSP70-2 gene 1267 A→G polymorphism in practically healthy and girls with vulvovaginitis did not differ reliably. The analysis of co-dominant model of inheritance showed a relative advantage of the AG- and GG-genotypes frequency in study group over AA-genotype by 17.5 and 16.5 times (χ^2=40.01 and χ^2=36.61, p<0.001).

Table 2: Distribution Hsp70-2 A-1267G gene polymorphism in vulvovaginitis adolescent girls

Genotypes, alleles of HSP70-2 gene, n	Groups, n (%)		OR [95% CI]	χ^2 p
	Study group, n=70 (%)	Control, n=30 (%)		
AA-genotype, n=2	2 (2.86)	0	-	-
AG-генотип, n=50	35 (50.0)	15 (50.0)	1.0 [0.43-2.35]	χ^2<1.0 p>0.05
GG-генотип, n=48	33 (47.14)	15 (50.0)	0.89 [0.38-2.10]	χ^2<1.0 p>0.05
χ^2p	χ^2<1.0 p>0.05	χ^2<1.0 p>0.05	-	-
A-allele, n=54	39 (27.86)	15 (25.0)	1.16 [0.58-2.31]	χ^2<1.0 p>0.05
G-allele, n=146	101 (72.14)	45 (75.0)	0.86 [0.43-1.72]	χ^2<1.0 p>0.05
OR [95% CI]	6.71 [3.98-11.31]	9.0 [3.94-20.57]	-	-
χ^2p	χ^2=54.91 p<0.001	χ^2=30.0 p<0.001	-	-

Note. OR – Odds Ratio; 95%CI OR - confidence interval of Odds Ratio.

The content of IL-1β, TNF-α, IL-10 in the blood plasma and sIgA in the vaginal secretion in patients and control group has been analyzed depending on the 1267 A→G polymorphism of HSP70-2 gene. The cytokines content level considered as: low (lower quartile of the experimental group) for IL-1β ≤6.4 pg/ml, TNFα - ≤6.6 pg/ml, IL-10- ≤5.17 pg/ml and sIgA - ≤1.0 ng/ml respectively (Table 3); high (the third quartile of the experimental group) for IL-1β - ≥7.98 pg/ml, TNFα - ≥7.40, IL-10 - ≥6.1 pg/ml and IL-13 sIgA - ≥5.3 ng/ml respectively. Patients with vulvovaginitis presented prevalence of a moderate and high production of pro-inflammatory cytokines (IL-1β i TNFα) 2.89 times (χ^2=33.03, p<0.001), that prove an increased activity of T-lymphocytes helpers of the 1st type (Th1) and macrophages.

Table 3: Levels of cytokines production in vulvovaginitis adolescent girls, M±S.D.

Cytokines	Control group	Study group	Levels of cytokines production in study group	
			Low production, n (%)	High, moderate production, n (%)
IL-1β, пг/мл	3.71±0.45	7.13±0.58 p<0.001	18 (25.71)	52 (74.29)
TNFα,пг/мл	5.04±0.25	7.12±0.42 p<0.001	18 (25.71)	52 (74.29)
IL-10, пг/мл	4.67±0.16	5.70±0.26 p<0.001	14 (20.0)	56 (80.0)
sIgA, нг/мл	1.40±0.41	3.64±0.30 p=0.001	17 (24.29)	53 (75.71)

On the other hand TNFα and IL-1β are the factors of activation, growth and maturation of T- and B-lymphocytes, NK-cells, fibroblasts, endothelial cells; IL-1β interacts with Th2 and induces the synthesis of IL-3, IL-4, IL-5, IL-6, IL-8, IF-γ, expression of IL-2 receptors, increases the secretion of antibodies by B-lymphocytes, causes chemotaxis of macrophages, neutrophils, promotes their migration through the vascular endothelium into the focus of inflammation where it activates synthesis of cytokines, prostaglandins, collagen and fibronectin, proteins of acute phase (C-reactive, mannose-binding etc.), reveals antipyretic action [18-23]. At the same time, we have found compensatory increase of the anti-inflammatory IL-10 content by 22.01% (p<0,001) via alternative ways. IL-10 is known as an Th1cells activity inhibitor, promotes the humoral response development, stimulates B-lymphocytes, mast cells, inhibits cellular immune response, decreases proliferation of T-cells and production of pro-inflammatory cytokines [19, 22, 23]. The number of individuals with a high and moderate production of IL-10 and secretory sIgA prevailed over those with a low production by 4 and 3.12 times (χ^2=50.4 i χ^2=37.03, p<0.001) respectively. In general the analyzed cytokines concentration was higher in girls with vulvovaginitis than in practically healthy subjects 1.22-2.6 times (p≤0.001) respectively (Table 3).

The cytokine production levels in girls with vulvovaginitis depending on the HSP70-2 gene genotypes are presented in the Table 4. The high-moderate production of IL-1β in the GG-genotype carriers was more frequent by 25.72% (87.88% against 62.16%, χ^2=6.04, p=0.014), than in A-allele (AA+AG-genotypes) carriers, as well as IL-10 – by 26.37% (93.94%

against 67.57%, χ^2=7.58, p=0.006, respectively). There was no reliable difference in TNF-α and sIgA production levels depending on the analyzed gene's genotypes.

Table 4: Cytokines production levels in vulvovaginitis adolescent girls depending on HSP70-2 A-1267G gene polymorphism

Cytokines	Cytokines production levels	Genotypes of HSP70-2 gene		
		AA, n=2 (%)	AG, n=35 (%)	GG, n=33 (%)
IL-1β	Low production, n=18 (%)	0	14 (40.0)	4 (12.12)
	High, moderate production, n=52 (%)	2 (100.0)	21 (60.0)	29 (87.88)
TNFα	Low production, n=18 (%)	0	12 (34.29)	6 (18.18)
	High, moderate production, n=52(%)	2 (100.0)	23 (65.71)	27 (81.82)
IL-10	Low production, n=14 (%)	0	12 (34.29)	2 (6.06)
	High, moderate production, n=56(%)	2 (100.0)	23 (65.71)	31 (93.94)
sIgA	Low production, n=17 (%)	0	10 (28.57)	7 (21.21)
	High, moderate production, n=53(%)	2 (100.0)	25 (71.43)	26 (78.79)

The blood plasma cytokines content (IL-1β, TNFα, IL-10) and sIgA vaginal secretion level depending on HSP70-2 gene allele condition is presented in the Table 5. The concentration of the immune system functioning analyzed markers in patients with vulvovaginitis prevailed reliably over healthy subjects one irrespective of the HSP70-2 gene genotypes (more in the GG-genotype carriers by the L-1β and TNFα levels). Although, in GG-genotype study group girls the content of IL-1β and TNFα was higher than in AA+AG-genotypes carriers by 12.13% (p=0.04) and 7.19 (p=0.055), respectively. The other indices did not differ between the groups depending on the HSP70-2 gene allele condition.

Table 5: Cytokines concentration production in vulvovaginitis adolescent girls depending on HSP70-2 gene allele's state, M±S.D.

Indexes	Control group	Genotypes of HSP70-2 gene, study group	
		AA+AG	GG-genotype
IL-1β, pg/ml	3.71±0.45	6.84±0.41 p<0.001	7.67±0.33 p<0.001 p_1=0.04
TNFα, pg/ml	5.04±0.25	6.95±0.22 p<0.001	7.45±0.26 p<0.001 p_1=0.055
IL-10, pg/ml	4.67±0.16	5.73±0.18 p<0.001	5.63±0.25 p<0.001
sIgA, ng/ml	1.40±0.41	3.65±0.45 p=0.005	3.62±1.05 p=0.048

Note. p – reliability of differences concerning control group; p_1 – reliability of differences concerning AA+AG-genotypes carriers.

Epidemiological analysis prove that HSP70-2 gene genotypes nor alleles are not additional risk factors of vulva and vagina inflammation in girls (Table 6). AG-genotype of HSP70-2 gene increases the probability of IL-1β lower production insignificantly 1.55 times [OR=3.5, 95% CI=0.93-13.12, p=0.056] and IL-10 – 1.71 times [OR =6.0, 95%CI =1.14-31.53, p=0.023], and on the contrary, the GG-genotype carriers have low chances for low IL-1β and IL-10 production [OR=0.29, 95%CI=0.08-1.07, p=0.057 and OR=0.17, 95% 95% CI=0.03-0.88, p=0.022] respectively. The HSP70-2 gene genotypes do not associate with the TNF-α and sIgA production levels and they are not risk factors of a high anti-inflammatory cytokine IL-10 production.

Table 6: Genotypes of HSP70-2 (rs1061581) gene polymorphism as a risk factors of vulvovaginitis in adolescent girls

Data	Potential Risk Factors			
	AA-, AG- genotypes	GG-genotype	A-алель	G-алель
RR	1.0	0.94	1.11	0.96
OR	1.0	0.89	1.16	0.86
95% CI RR	0.65-1.53	0.61-1.46	0.67-1.86	0.80-1.15
95% CI OR	0.43-2.35	0.38-2.10	0.58-2.31	0.43-1.72
p	>0.05	>0.05	>0.05	>0.05

Note. RR – Risk Ratio; OR – Odds Ratio; 95% CI RR, OR - confidence interval of RR, OR.

Population and racial analysis showed that the AA- minor genotype frequency of HSP70-2 gene was lower among the examined individuals (zero in the control, 2,86% - in study group) than in Caucasian populations an average, giving evidence of a high populations heterogeneity and inconsistency after polymorphic locus, nosology, etc. But allele distribution in our study in general corresponded to Caucasian race and did not differ reliably (P_A=0.25-0.28 vs P_A=0.33-0.71 and P_G=0.72-0.75 vs P_G=0.67-0.87). At the same time the A-allele frequency in our study was high than in Afro-American population (P_A=0.25-0.28 vs P_A=0-0.13), and reliably lower than in the Asians (P_A=0.44-0.48, p<0.05) respectively [24].

4. Conclusion

The homozygous mutation of HSP70-2 gene (rs1061581) in juvenile girls of Northern Bukovina (Western Ukraine) with vulvovaginitis is found in 2.86% cases, and is absent in healthy. Wild G allele of HSP70-2 gene present in every second teenager. The vulvovaginitis course in juvenile girls is accompanied by an increase of cellular immunity activity with prevailing of high and moderate production of pro-inflammatory cytokines (IL-1β and TNFα) 2.89 times (χ^2=33.03, p<0.001) (especially in the GG-genotype carriers by 12.13% (p=0.04) and 7.19 (p=0.055), respectively) with compensatory increase of humoral immunity activity by synthesis of anti-inflammatory IL-10 by 22.01% (p<0.001)

and activation of local immunity of the vaginal mucosa by the secretory sIgA increase 2.6 times ($p<0.001$) respectively, which did not depend on allele condition of HSP70-2 gene.

Genotypes and alleles of HSP70-2 gene (rs1061581) are not additional risk factors of the vulva and vagina inflammation in juvenile girls of Northern Bukovina. AG-genotype of HSP70-2 gene increases chances to lower IL-10 production by 1.71 times [OR=6.0, p=0.023] and, boundary, pro-inflammatory IL-1β by 1.55 times [OR=3.5, p=0.056] with low probability in the GG-genotype carriers [OR=0.17, p=0.022 and OR=0.29, p=0.057] respectively, which indicates a susceptibility to worse immune defense in the AG-genotype carriers.

Conflict of Interest: None declared.

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