EPIGENETIC MARKERS OF ASTHMA: A STUDY OF METHYLATION PATTERNS OF GENES INVOLVED IN DRUG METABOLISM

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Abstract. Asthma is a common chronic multifactorial respiratory disease. Genes involved in the metabolism of drugs used for asthma management play an important role in asthma development. The aim of the study was to analyze the methylation of the promoter regions of *AOC1, GLCCI1* and *ARG2* genes involved in the metabolism of drugs used for asthma treat-ment in asthma patients and controls from the Republic of Bashkortostan. DNA was extracted from peripheral blood sam-ples of 157 asthma patients and 155 control subjects. Methylation-Sensitive High Resolution Melting analysis and se-quencing of bisulfite-treated genomic DNA were applied to estimate the degree of methylation. Analysis of the methylation status of promoter region of the *AOC1* gene revealed a higher frequency of full methylation (100%) of the studied region in patients with severe and moderate asthma than in controls (38.61%; p = 0.002; OR = 2.58; 95%CI 1.4-4.75). A signifi-cantly higher level of promoter methylation of the *GLCCI1* gene was found in patients with severe and moderate asthma compared to control group (p = 0.01; OR = 3.1; 95%CI 1.22-7.88). A low level of promoter methylation of the *ARG2* gene was determined in both analyzed groups of patients and controls. The results of MS-HRM analysis were confirmed by bisulfite sequencing of analyzed samples. Thus, this study revealed differences in the level of methylation of promoter regions of *AOC1* and *GLCCI1* genes between samples of asthma patients and controls. The results of the study expand general understanding of the possible contribution of DNA methylation to asthma development.

Keywords: bronchial asthma, DNA methylation, gene.

List of Abbreviations

DNA – deoxyribonucleic acid

MS-HRM – Methylation-Sensitive High Resolution Melting

Introduction

Asthma is a chronic disease of the respiratory tract, the development of which is determined by a complex interaction of genetic, epigenetic and environmental factors. The heritability of asthma is up to 90% in children and to 74% in adults (Hernandez-Pacheco *et al.*, 2019). Airway inflammation is the main pathogenetic link determining asthma development and course. Genes involved in the metabolism of antiasthmatic drugs play an important role in asthma pathogenesis. The above group includes genes encoding specific receptors regulating inflammation, cell proliferation and differentiation (NR3C1, ADRB2), as well as proteins involved in various stages of inflammatory mediator biosynthesis (HRH1, HRH2, HRH3, HRH4, LTC4S, ALOX5, LTA4) and others.

The study of the epigenetic mechanisms of gene regulation in allergic diseases is one of the most relevant fields of modern research. Epigenetic mechanisms can influence on gene expression without altering the DNA sequence and provide gene-environment interactions. Specifically, epigenome modifications mediate endogenous or exogenous environmental influences for allergic development (Legaki *et al.*, 2022). The respiratory system is constantly exposed to the environment (chemicals, dust, bacteria, viruses, etc.), the epigenome of airway cells is susceptible to dynamic changes which can consequently affect a gene expression.

Several studies exploring the role of DNA methylation in the pathogenesis of allergic diseases were performed. A number of large-scale studies of DNA methylation profiles in asthma patients revealed a large amount of differentially methylated regions associated with asthma development. For example, 589 differentially methylated CpG sites in cord blood mononuclear cells in newborns from mothers with asthma were identified (De Vries *et al.*, 2017). In another study, 186 differentially methylated CpG sites in genes involved in immune response, airway obstruction and cell adhesion, associated with risk of asthma and atopy were revealed in the nasal epithelium of African American children (Yang *et al.*, 2017). A whole-epigenome study of the nasal epithelium of children from different backgrounds identified 285 asthma-associated CpG sites (Cardenas *et al.*, 2019). A meta-analysis of EWAS asthma studies in peripheral blood leukocytes showed that CpG sites most significantly associated with asthma development were located near *CAMK1D* and *TIGIT* genes involved in the development of inflammation (Jiang *et al.*, 2021). EWAS of DNA blood revealed 5 CpG sites associated with different measures of lung function in Hispanic asthma patients (Herrera-Luis *et al.*, 2022). Whole-genome bisulfite sequencing of peripheral blood found 158 differentially methylated regions between asthma patients and controls. Thürmann and colleagues showed that hypomethylation predominantly affects enhancer regions of key genes regulating the immune response, such as IL4, IL5RA and EPX (Thürmann *et al.*, 2023).

Only a few studies were conducted to analyse the role of DNA methylation in the development of allergopathologies in Russia. Bystritskaya E.P. et al. found a higher number of unmethylated sites of the promoter regions of *TLR2* and *TLR4* genes in the respiratory epithelial cells of children with severe and moderate asthma than in controls (Bystritskaya *et al.*, 2019). The differential methylation level of 26 genes whose protein products functions were associated with the regulation of fibroblasts, dendritic cell migration and activation of general immunity (*ALPK2, CLNK, ARHGEF4, MIR1178, VPS37C, GRIP2, SLC47A2, TCF12, ZBTB20* и др.) was revealed in skin biopsy of atopic dermatitis patients compared to biopsy of unaffected skin (Smolkina *et al.*, 2021). Seventeen differentially methylated CpG sites associated with atopic dermatitis development were found (Bystritskaya *et al.*, 2022).

Previous our studies of genes involved in the metabolism of drugs used for asthma therapy revealed an association of the rs37973**G* allele of the *GLCCI1* gene with uncontrolled asthma in Tatars (Fedorova *et al.*, 2019). In Russians, rs17249437**TT* and rs3742879**GG* genotypes of the *ARG2* gene as well as the rs1049793**C* allele of the *AOC1* gene were associated with a significant decrease of pulmonary function (Savelieva O.N. *et al.*, 2020 a,b). Based on the above, the promoter regions of *GLCCI1, AOC1* and *ARG2* genes involved in the metabolism of antiasthmatic drugs were selected for methylation analysis to identify potential epigenetic markers affecting the development and progression of asthma in individuals from the Republic of Bashkortostan.

The aim of this study was to analyze the methylation of promoter regions of *AOC1*, *GLCCI1* and *ARG2* genes involved in the metabolism of drugs used to asthma treatment in individuals with asthma and control subjects from the Republic of Bashkortostan.

Materials and Methods

DNA samples of 312 unrelated individuals aged from 2 to 18 years old of Russian and Tatar ethnicity living in the Republic of Bashkortostan were used as study material. The group of patients included 157 individuals with asthma from the BSMU Clinic and the State Budgetary Institution of the Ufa Russian Children's Hospital and the control group included 155 practically healthy individuals (without bronchopulmonary and allergic diseases). All study participants (or their official representatives) signed an informed consent approved as part of the study protocol by the ethical committee prior to inclusion in the study. The study conformed to the standards set by the latest revision of the Declaration of Helsinki and the procedures were approved by the Bioethical

Committee of the IBG UFRC RAS (protocol № 7 of 10.02.2011).

DNA was isolated from peripheral blood by phenol-chloroform extraction. Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, USA). The methylation levels of the promoter regions of the *AOC1* (GRCh38:7: 150824654-150824801), *GLCCI1* (GRCh38:7:7969920-7970072) and *ARG2* (GRCh38:14:67619480-67619620) genes were analyzed by Methylation-Sensitive High Resolution Melting (MS-HRM) analysis. Primers were selected using Methyl Primer Express Software v1.0 (Table 1). MeltDoctor HRM Master mix PCR mix (Applied Biosystems, USA) was used for MS-HRM analysis.

Bisulfite-converted samples of methylated (100%) and unmethylated (0%) DNA (Zymo Research, USA) were diluted at specific percentages (0/100, 5/95, 10/90, 25/75, 50/50, 75/25, 90/10, 100/0) to obtain standard controls for methylation levels resulting in methylation levels of the control samples of 0%; 5%; 10%; 25%, 50%, 75%, 90% and 100%, respectively. The methylation level of the tested samples was determined by comparing their melting curves with the melting curves of control samples set as Auto Standard Curve in the protocol of the LightCycler® 96 Roche instrument (LightCycler® 96 Roche, Germany). The MS-HRM results were confirmed by sequencing of bisulfite-converted DNA on an ABI PRISM 3500 instrument (Applied Biosystems, USA).

Results

The methylation analysis of the promoter region of the *AOC1* gene (GRCh38:7: 150824654-150824801, 7 CpG sites) was performed in asthma patient and control group individuals from the Republic of Bashkortostan. MS-HRM analysis of the promoter region of the *AOC1* gene revealed that the level of methylation of this region in asthma patients and controls was ranged from 50% to 100% (Fig. 1, Table 2).

Table 1

The characteristics of amplification conditions for the investigated gene regions

Gene	Nucleotide sequence	Annealing temperature
AOC1	5' AATTTGATTTTGGGAAGATAGG 3' 5' CCCTTCAAAACTACATCTCGTA 3'	60°
GLCCI1	5' AAGTGGTTTTGGGAATTTTATTTT 3' 5' AACCAACTCTTAAACTAAAAAACCTCT 3'	62°
ARG2	5'AAGTAGGTGGATTTTGGTTTTG 3' 5'ACCCATACCAAATTCCTAAACTT 3'	62°

Table 2

Results of MS-HRM analysis of the promoter region of the *AOC1* gene

Methylation level, %	Studied group		
	General group of asthma patients	Group of patients with severe and moderate asthma	Control group
	N (%)	N (%)	N (%)
100	59 (49.58) p = 0.10	**47 (61.84)** **p = 0.002** **OR = 2.58** **95%CI 1.40-4.75**	39 (38.61)
90	46 (38.66) p = 0.08	**24 (31.58)** **p = 0.01** **OR = 0.45** **95%CI 0.24-0.84**	51 (50.5)

End table 2

Methylation level, %	Studied group		
	General group of asthma patients	Group of patients with severe and moderate asthma	Control group
	N (%)	N (%)	N (%)
75	14 (11.76) p = 0.66	5 (6.58) p = 0.61	10 (9.9)
50	-	-	1 (0.99)
N	119	76	101

Note: N – group size; frequency of methylation levels in individuals is given in parentheses, %; p - level of statistical significance differences in methylation level frequencies between patient and control groups; OR - odds ratio and 95%CI - confidence interval, given at $p < 0.05$

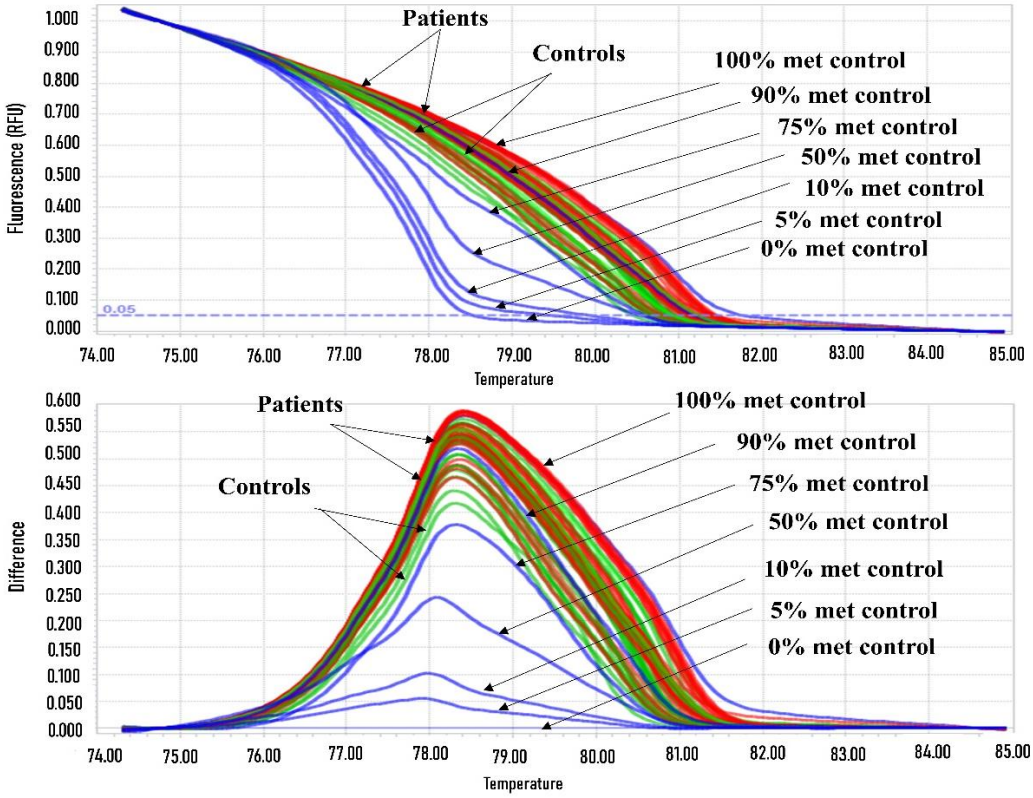

Fig. 1. MS-HRM analysis of the promoter region of the *AOC1* gene in the groups of asthma patients and controls. Melting curves of control standards with different methylation levels are blue (0% met, 5% met; 10% met, 50% met, 75% met, 90% met, 100% met); melting curves of asthma patients are red; melting curves of individuals from the control group are green

Comparative analysis revealed no statistically significant differences in the occurrence frequency of different levels of methylation in the promoter region of the *AOC1* gene between the whole group of asthma patients and controls ($p > 0.05$). However, patients with severe and moderate asthma were detected to have a higher incidence of complete methylation of the study region of the *AOC1* gene in cases (61.84%) compared to control samples (38.61%; $p = 0.002$; OR = 2.58; 95%CI 1.4-4.75).

In the next step, sequencing of sodium bisulfite-treated DNA samples from asthma patients with different methylation levels and the control methylation standards was performed to confirm the results obtained by MS-HRM analysis. The high level of methylation of the analyzed region of the *AOC1* gene was confirmed

by DNA sequencing of the studied asthma patients (Fig. 2).

Analysis of the methylation status of the promoter region of the *GLCCI1* gene in samples ofasthma patients and controls from the Republic of Bashkortostan (GRCh38:7:7969920-7970072, 7 CpG sites) was carried out. The level of *GLCCI1* gene methylation in groups of asthma patients and controls was ranged from 0% to 75% according to MS-HRM analysis (Fig. 3, Table 3). A significantly higher incidence of 50% methylation level was found in individuals with severe and moderate asthma (20.9%) compared to controls (7.84%; p = 0.01; OR = 3.1; 95%CI 1.22-7.88). The subsequent bisulfite sequencing of the analyzed samples confirmed the results found by MS-HRM analysis. Figure 4 shows the results of DNA sequencing of asthma patients with 40%-50% methylation level of CpG sites located in the investigated promoter region of the *GLCCI1* gene and 100% methylated DNA standard (Fig. 4).

The analysis of methylation level of 7 CpG sites in promoter region of *ARG2* gene (GRCh38:14:67619480-67619620) in patients and control group individuals from the Republic of Bashkortostan revealed a low level of methylation of all analyzed CpG sites of this gene in both samples (0%-2%).

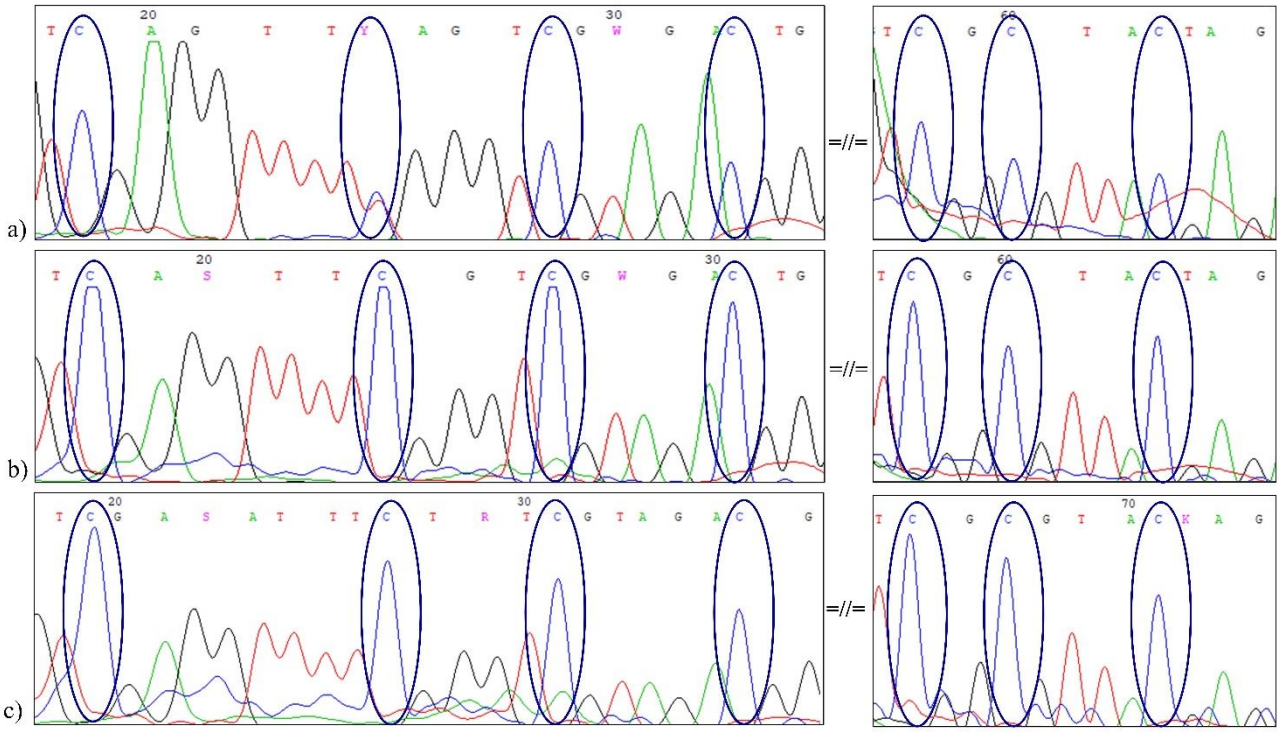

Fig. 2. The sequence of the promoter region of the *AOC1* gene in asthma patients with high methylation level according to MS-HRM analysis (a, b) and 100% methylated control DNA (c)

Table 3

Results of MS-HRM analysis of the promoter region of the *GLCCI1* gene

Methylation level, %	Studied group		
	General group of asthma patients	Group of patients with severe and moderate asthma	Control group
	N (%)	N (%)	N (%)
0	**1 (0.92) p = 0.03 OR = 0.11**	-	8 (7.84)

End table 3

Methylation level, %	Studied group		
	General group of asthma patients	Group of patients with severe and moderate asthma	Control group
	N (%)	N (%)	N (%)
	95%CI 0.01-0.89		
5	16 (14.68) p = 0.99	10 (14.93) p = 0.99	15 (14.71)
10	32 (29.36) p = 0.87	22 (32.84) p = 0.74	31 (30.39)
25	42 (38.53) p=0.96	21 (31.34) p = 0.36	39 (38.24)
50	18 (16.51) p = 0.06	**14 (20.9)** **p = 0.01** **OR = 3.1** **95%CI 1.22-7.88**	8 (7.84)
75	-	-	1 (0.98)
N	109	67	102

Note: N – group size; frequency of methylation levels in individuals is given in parentheses, %; p – level of statistical significance differences in methylation level frequencies between patient and control groups; OR – odds ratio and 95%CI – confidence interval, given at $p < 0.05$

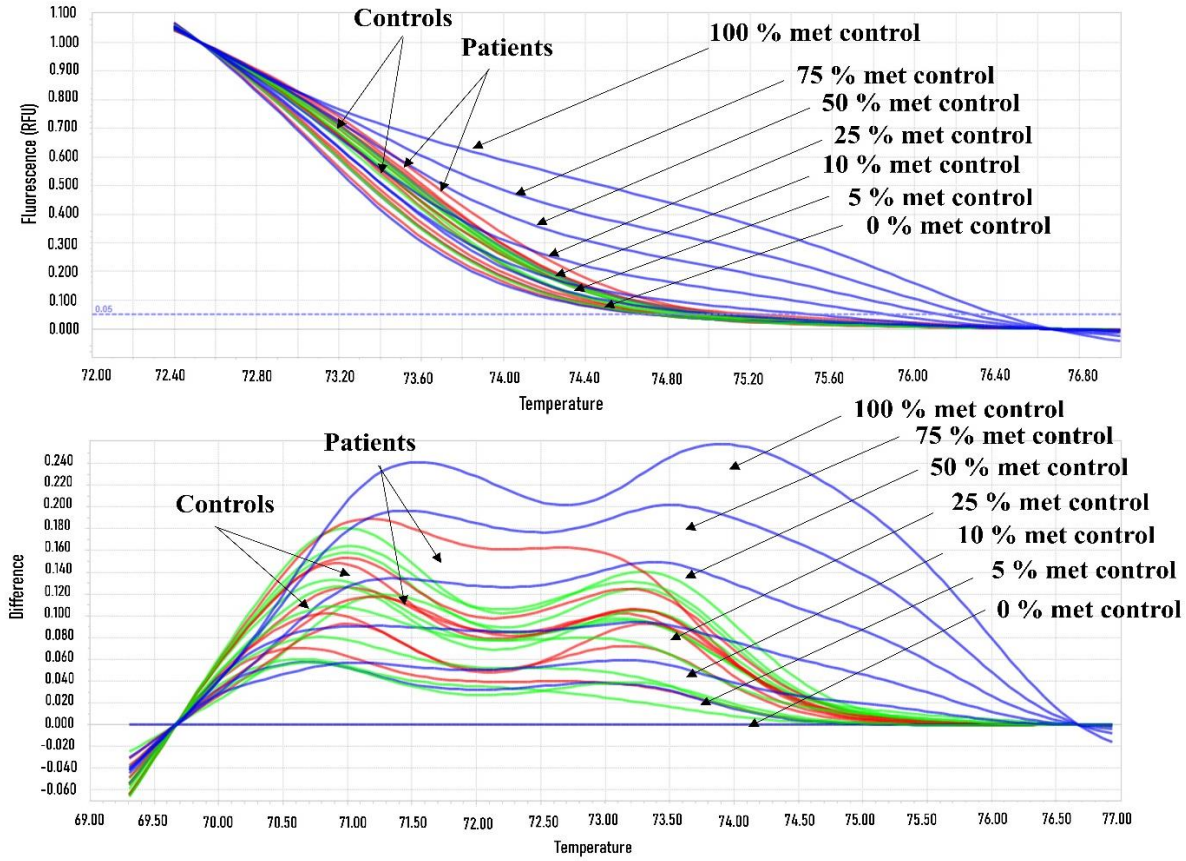

Fig. 3. MS-HRM analysis of the promoter region of the *GLCCI1* gene in the groups of asthma patients and controls. Melting curves of controls with different methylation levels are blue (0% met, 5% met; 10% met, 50% met, 75% met, 90% met, 100% met); melting curves of asthma patients are red; melting curves of individuals from the control group are green

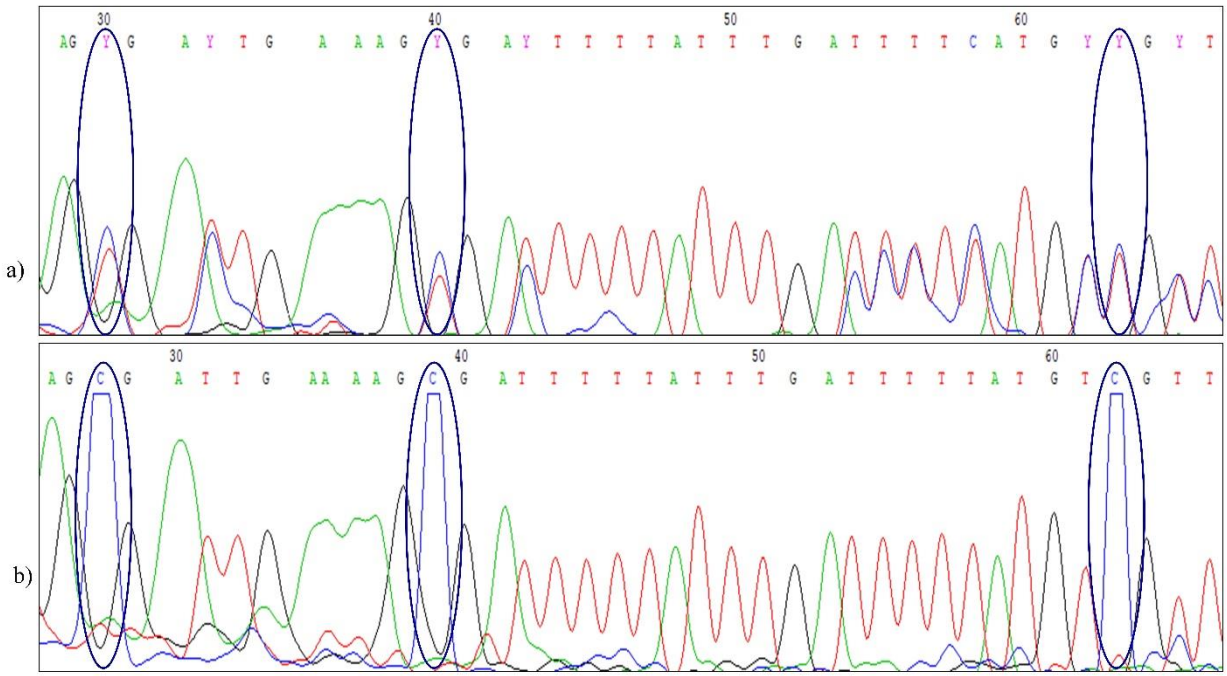

Fig. 4. The sequence of the promoter region of the *GLCCI1* gene in asthma patient (a) and 100% methylated control DNA (b)

Discussion

DNA methylation is one of the most important mechanisms of epigenetic regulation of gene expression. DNA methylation is assumed to be a promising biomarker of allergic diseases and certain immune phenotypes (De Vries *et al.*, 2017; Cardenas *et al.*, 2019; Jiang *et al.*, 2021; Herrera-Luis *et al.*, 2022; Thürmann *et al.*, 2023). The methylation analysis of the promoter regions of *AOC1*, *GLCCI1* and *ARG2* genes in the peripheral blood of children living in the Republic of Bashkortostan was carried out in our study.

The amine oxidase 1 enzyme encoded by the *AOC1* gene catalyzes the degradation of the inflammatory mediator histamine and compounds involved in the formation of immune and allergic response (Kucher & Cherevko, 2018). Numerous studies, including our own, identified polymorphisms in the *AOC1* gene associated with allergic rhinitis (Meza-Velázquez *et al.*, 2016). Additionally, it was showed that these polymorphisms were associated with the histamine response in asthma patients (Jones *et al.*, 2017) and play a role in asthma development, contributing to a marked decline in pulmonary function indices in Russians (Savelieva *et al.*, 2020a). In the current study, we found that complete methylation of the promoter region of the *AOC1* gene is more frequent in patients with severe and moderate asthma compared to the control group. The analyzed region of the *AOC1* gene contains a promoter-like element EH38E2600888 (PLS, promoter-like signature) associated with the multifunctional transcription factor CTCF, known to play an important role in the control of gene expression in vertebrates. Promoter-like elements are cis-regulatory elements with high levels of signaling to DNase and H3K4me3, typically localized within 200 bp of the transcription start site (Zhu *et al.*, 2024). According to ORegAnno and Jaspar data, it was confirmed that the binding sites of a wide range of other transcription factors such as TBX20, TBX18, TBX21, TBX5, TCF7L1 etc. were located in the study region (https://genome.ucsc.edu). Whole-genome bisulfite sequencing of peripheral blood cells from healthy individuals revealed that the methylation level of the *AOC1* gene region investigated in this study varies from 50% to 100%, with an average methylation level of

80% (Li *et al.*, 2010). Whole-epigenome analysis showed lower levels of methylation of cg26475742 localized in the *AOC1* gene in primary airway epithelial cells from asthma patients than in controls of different backgrounds (Nicodemus-Johnson *et al.*, 2016). EWAS of nasal epithelial cells from patients with allergic diseases and control individuals of different origin revealed 191 differentially methylated CpG sites associated with changes in exhaled nitric oxide fraction levels between the studied cohorts, among which cg14909495 localized in the *AOC1* gene (Cardenas *et al.*, 2019). The results obtained in this study are partially consistent with previous work indicating differences in the methylation profile of individual CpG sites of the *AOC1* gene between patients with allergic pathologies and controls.

The glucocorticoid-induced transcript 1 gene *GLCCI1* is located in the 7p21 region and includes eight exons, it is actively expressed in the lung and in immune cells (Salhi *et al.*, 2021). GLCCI1 protein known to be involved in the glucocorticoid-glucocorticoid receptor interaction cascade, producing an anti-apoptotic effect against T cells (Kiuchi *et al.*, 2019). The rs37973 polymorphism of the *GLCCI1* gene was associated with asthma development and the efficacy of inhaled GCS therapy in asthma patients (Tantisira *et al.*, 2011; Feng *et al.*, 2023). Hu C. et al. revealed that changes in GLCCI1 mRNA expression were positively correlated with spirography measures (FEV1) in Chinese individuals with asthma treated with inhaled GCS (Hu *et al.*, 2016). Previously, we also found an association of the rs37973**G* allele of the *GLCCI1* gene with uncontrolled asthma in Tatars (Fedorova *et al.*, 2019). The promoter region of the *GLCCI1* gene includes binding sites for various transcription factors, including the multifunctional transcription factor (CTCF) involved in activation and repression of transcription (https://www.ensembl.org), as well as a number of other transcription factors (TBX21, TBX20, TBX2, EOMES, TBR1, TBX18, TBX3, Tbx6, ZKSKAN5) (https://genome.ucsc.edu). According to the literature, a lower level of methylation of the 5 CpG sites of the *GLCCI1* gene in peripheral blood cells of Chinese asthma patients than in controls was found to be associated with a reduced level of expression of this gene. Different types of T cells (Th1, Th2, Th17) and B cells are involved in the asthma development. Therefore, it is suggested that changes in the methylation status or expression level of the *GLCCI1* gene may affect the functioning of T- or B-lymphocytes and asthma progression in general (Jiang *et al.*, 2021). A number of previous studies reported that the methylation level of one of the CpG sites localized in the promoter region of the *GLCCI1* gene ranged from 66% to 81% (Li *et al.*, 2010). In the current study a significant difference in the degree of methylation of the analyzed CpG sites of the *GLCCI1* gene between patients with severe and moderate asthma and controls was found.

The *ARG2* gene encodes one of the arginase type II isoenzymes overexpression of which promotes airway remodeling and cell proliferation. Vonk J.M. et al. found that the rs3742879**AA* genotype of the *ARG2* gene was associated with increased bronchial hyperresponsiveness in asthma patients living in the Netherlands (Vonk *et al.*, 2010). The combination of NOS2A*(CCTTTT)nS/L and rs3742879*AA genotypes of the *ARG2* gene was associated with risk of asthma development in women from Russia (Batozhargalova *et al.*, 2017). The combination of NOS2A*(CCTTTT)nS/L and rs3742879*AA genotypes of the *ARG2* gene was associated with asthma in women from Russia (Batozhargalova *et al.*, 2017). In our previous studies, the association of rs17249437**TT* and rs3742879**GG* genotypes of the *ARG2* gene with a significant decrease of FEV1 and MEF25 in Russians was established (Savelieva *et al.*, 2020b). In the present study, a low level of methylation of all analyzed CpG sites of the promoter region of the *ARG2* gene was detected in both asthma patients and controls. According to UCSC browser data, the promoter-like element EH38E1722878 and the proximal enhancer-like region EH38E1722877 are located in the investigated region of the *ARG2* gene, comprising binding sites for the transcription

factor CTCF and other transcription factors such as ETV5, EFR, ETV2, ASCL1, TCF3, SNAI1, TCF12, TCF4, SNAI3, Tfcp211, Handl, Zfx, ZNF449, MAF, Mafg, ZNF449 et al. (https://genome.ucsc.edu). A low level of methylation of 13 investigated CpG sites of the promoter region of the *ARG2* gene was found in buccal epithelial cells of allergic patients from China (5-methylcytosine 5mC<3%) (Ji et al., 2021), which is consistent with the results obtained in our current study indicating a low level of methylation of this gene in patients with allergic diseases.

Conclusion

Thus, in the present study we analyzed the methylation status of particular parts of *GLCCI1*, *AOC1* and *ARG2* genes located in the regions directly regulating transcription (promoters, enhancers, transcription factor binding sites) in the peripheral blood of asthma patients and control subjects from the Republic of Bashkortostan. Statistically significant differences in the level of methylation of *GLCCI1* and *AOC1* promoter regions between the patients with severe and moderate asthma compared to the control sample were revealed. In our previous studies, the association of the rs37973**G* allele of the *GLCCI1* gene with uncontrolled asthma in Tatars was found. In Russians, the associations of the rs104979793**CC* genotype and the rs104979793**C* allele of the *AOC1* gene with asthma and a significant decrease in pulmonary function measures and the rs17249437**TT* and rs3742879**GG* genotypes of the *ARG2* gene with a significant decrease of FEV1 and MEF25 were established. The combination of the obtained results and previously published data suggests a certain contribution of the studied genes in asthma development.

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The authors declare that they have no competing interests.

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