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Relationship of the expression of calcium-handling proteins in the sarcoplasmic reticulum with polymorphic variants of their genes and with structural and functional parameters of the heart in patients with atrial fibrillation

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ABSTRACT

Aim. To investigate the relationship between the expression of Ca^{2+} handling proteins of the sarcoplasmic reticulum, polymorphic variants of their genes, and structural and functional parameters of the heart in patients with atrial fibrillation (AF).

Materials and methods. The study included patients with AF. The patients underwent radiofrequency ablation, during which a myocardial biopsy was taken. The patients underwent echocardiography (EchoCG) before surgery. Polymorphic variants rs1860561 of the *ATP2A2* gene and rs6684209 and rs7521023 of the *CASQ2* gene were determined in the patients by real-time polymerase chain reaction (PCR), and the level of expression of SERCA2a and CASQ2 proteins in the myocardium was detected by immunoblotting.

Results. Carriers of the GG genotype at rs1860561 of the *ATP2A2* gene and CC genotype at rs6684209 of the *CASQ2* gene were characterized by significantly higher expression of the corresponding proteins. Using cluster analysis, we identified groups of patients by the level of SERCA2a and CASQ2 expression: group 1 – patients with low protein content; group 2 – patients with high protein content. According to clinical and anamnestic parameters, the patients in the selected groups were homogeneous. In patients with high SERCA2a levels, the end systolic and diastolic volumes of the left ventricle (LV) were significantly higher than those in patients with low levels of this protein. The rates of early (peak E) and late left ventricular diastolic filling (peak A) were significantly lower in the group with high SERCA2a expression. A comparative analysis of EchoCG data of patients distributed by the level of CASQ2 expression in the myocardium did not reveal significant differences between the groups.

Conclusion. The polymorphic variant rs1860561 of the *ATP2A2* gene and rs6684209 of the *CASQ2* gene can modulate the level of SERCA2a and CASQ2 expression. SERCA2a expression is associated with the functional and structural parameters of the heart in patients with AF.

Keywords: atrial fibrillation, Ca^{2+}-ATPase of the sarcoplasmic reticulum, calsequestrin, polymorphic variants of genes, echocardiography

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at the Cardiology Research Institute, Tomsk NRMC (Protocol No. 139 of 18.11.2015).

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Сопряженность экспрессии кальций-транспортирующих белков саркоплазматического ретикулума с их полиморфными вариантами генов и структурно-функциональным состоянием сердца пациентов с фибрилляцией предсердий

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РЕЗЮМЕ

Цель. Исследовать взаимосвязь между экспрессией Ca2+-транспортирующих белков саркоплазматического ретикулума, полиморфными вариантами их генов и структурно-функциональным состоянием сердца пациентов с фибрилляцией предсердий (ФП).

Материалы и методы. В исследование включили пациентов с ФП. Больным проведена радиочастотная аблация, во время которой была взята биопсия миокарда. Пациентам проводили эхокардиографию (ЭхоКГ) до оперативного вмешательства. У больных определены полиморфные варианты rs1860561 гена *ATP2A2* и rs6684209, rs7521023 гена *CASQ2* методом полимеразной цепной реакции в режиме реального времени и уровень экспрессии белков SERCA2a и CASQ2 в миокарде методом иммуноблоттинга.

Результаты. Для носителей генотипов GG rs1860561 гена *ATP2A2* и CC rs6684209 гена *CASQ2* характерны значимо более высокие экспрессии соответствующих белков. С помощью кластерного анализа были выявлены группы пациентов по уровню экспрессии SERCA2a и CASQ2: 1 – пациенты с низким содержанием белков; 2 – с высоким содержанием белков. По клинико-анамнестическим показателям пациенты отобранных групп оказались практически однородны. У пациентов с высоким уровнем SERCA2a величины конечного систолического и диастолического объемов левого желудочка (ЛЖ) были значимо больше, чем таковые у больных с низким уровнем этого белка. Скорости раннего (пик Е) и позднего диастолического наполнения (пик А) ЛЖ были статистически значимо ниже в группе с высоким уровнем экспрессии SERCA2a. Сравнительный анализ данных ЭхоКГ пациентов, распределенных по уровню экспрессии CASQ2 в миокарде, не выявил значимых различий между группами.

Заключение. Генотипы rs1860561 гена *ATP2A2* и rs6684209 гена CASQ2 могут модулировать уровень экспрессии SERCA2a и CASQ2. Экспрессия SERCA2a сопряжена с функционально-структурными показателями сердца пациентов с ФП.

Ключевые слова: фибрилляция предсердий, Ca^{2+}-АТФаза саркоплазматического ретикулума, кальсеквестрин, полиморфные варианты генов, эхокардиография

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом НИИ кардиологии Томского НИМЦ (протокол № 139 от 18.11.2015).

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INTRODUCTION

In recent decades, the growth in the atrial fibrillation (AF) prevalence among the population of developed countries of the world has increased by 2–3 times [1]. According to the Framingham Heart Study, AF patients have a 1.5–2 times increased risk of annual mortality compared with the general population [2]. Despite significant advances in the study of AF mechanisms, the existing AF treatment standards have limited effectiveness [3]. It has been established that the molecular mechanisms of the triggered activity of cardiomyocytes are caused by impaired intracellular homeostasis of calcium ions [4]. One of the key functional proteins providing the transport of calcium ions in the cell is Ca^{2+}ATPase (SERCA2a) of the sarcoplasmic reticulum (SR). This protein is responsible for reuptake of calcium ions from the myoplasm into the SR [5]. A protein called calsequestrin (CASQ2) is responsible for binding of calcium ions inside the SR. In addition, CASQ2 affects the stability of the ryanodine receptor structure in the SR [6]. This suggests that CASQ2 is involved in the development of diastolic calcium leak.

The significance of the functional state of SERCA2a and CASQ2 in the mechanisms of AF initiation and maintenance has been shown both in experimental studies [7, 8] and in clinical trials [9, 10]. At the same time, the presence of AF is associated with both low and high levels of SERCA2a in the myocardium [9, 10]. Such a difference in the results may be due to the peculiarities of the examined samples of patients and, in particular, due to the different genetic variants of these proteins. Indeed, it was found that the genes encoding SERCA2a and CASQ2 have stable polymorphic variants which can affect the functional characteristics of these proteins. It was discovered that the presence of the polymorphic variant rs1860561 of the Ca^{2+}ATPase gene (*ATP2A2*) may be associated with a lower risk of life-threatening arrhythmias [11]. The involvement of CASQ2 in provoking sudden cardiac arrest due to ventricular arrhythmias may be associated with the polymorphic variant rs7521023 of the *CASQ2* gene [12]. However, the available data are insufficient to say whether these proteins and their genes are associated with the structural and functional parameters of the heart in AF patients.

The aim of the study was to assess the relationship of the expression of Ca^{2+}ATPase and calsequestrin, as well as the presence of polymorphic variants rs1860561 of the *ATP2A2* gene and rs7521023 and rs6684209 of the *CASQ2* gene with the structural and functional parameters of the heart in patients with AF.

MATERIALS AND METHODS

An observational, cross-sectional, uncontrolled study was carried out. The study included 45 patients with AF. The study was carried out in accordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee at the Cardiology Research Institute of Tomsk NMRC. All patients signed an informed consent to participate in the study. The average age of the patients was 43 [39; 48] years. The duration of AF was 3 [2.0– 4.0] years. As antiarrhythmic therapy, the patients received: amiodarone – 11 (24%), sotalol – 6 (13%), propafenone – 11 (24%), beta blockers – 7 (16%), and allapinin – 2 (4%).

At the time of hospitalization, 26 (58%) patients were receiving anticoagulant therapy, and 7 (16%) patients were receiving antiplatelet therapy. The incidence of hypertension was 31% (n = 14), and the incidence of coronary artery disease was 9% (n = 4). Upon admission, the patients underwent a general clinical examination, standard 12-lead electrocardiography (ECG), and transthoracic and transesophageal echocardiography (EchoCG). The patients included in the study had functional class (FC) 0–II chronic heart failure (CHF), according to the New York Heart Association (NYHA) functional classification. The exclusion criteria were the following: FC III–IV CHF (NYHA), heart valve disease, as well as systemic, acute, and chronic inflammatory diseases, and cancer.

To assess intracardiac hemodynamic parameters, the patients underwent M-mode and 2D echocardiography from standard positions on the Philips En Visor CHD ultrasound machine (Netherlands). 24 (15.0%) patients had left ventricular hypertrophy, and in 65 patients [61; 67] % of patients, left ventricular ejection fraction was detected.

The patients underwent radiofrequency ablation (RFA), during which myocardial biopsies were taken to exclude viral myocarditis (the apex of the right ventricle, the right ventricular outflow tract and the interventricular septum). AF of unknown etiology was an indication for biopsy. The patients had no complications after the biopsies were taken.

A part of the biopsy sample (1–2 mg) was used to determine the SERCA2a and CASQ2 levels by immunoblotting. The tissue was homogenized (Bullet Blender, Next Advance Inc., USA) in a lysis buffer. Cell membranes were disrupted using ultrasound (Sonopuls, Bandelin). The homogenates were centrifuged

at 16,000g and 4° C for 25 min. The proteins were separated in polyacrylamide gel electrophoresis. Semidry electroblotting (BlueBlot SD, SERVA) was used to transfer proteins onto the nitrocellulose membrane. We used primary SERCA2a (1 : 2,000) and CASQ2 (1 : 2,000) monoclonal antibodies and alkaline phosphatase-conjugated secondary antibodies. BCIP / NBT was used to detect proteins. The level of total protein in the sample was determined on the spectrophotometer at 280 nm corrected for the presence of nucleic acids (260 nm) (NanoVue™, ThermoFisher Scientific). The content of target proteins was calculated relative to the expression of the β-actin protein. All reagents used in the study were manufactured by Sigma-Aldrich (USA).

Genomic DNA was isolated from blood leukocytes of the patients according to the manufacturer's protocol (Promega, USA). The polymorphic variant rs1860561 (110345436G>A in the intron) of the Ca^{2+}ATPase gene (*ATP2A2*) was determined. For the CASQ2 (*CASQ2*) gene, the polymorphic variants rs6684209 (115707991C>T in the intron) and rs7521023 (115700759G>A in the 3′-UTR) were identified. The study was carried out using a real-time polymerase chain reaction (PCR) (DT-96, DNA-Technology, Russian Federation). Primers and fluorescent probes (FAM and HEX) (TestGen, Russian Federation) were used for DNA amplification. The distribution of genotype frequencies was checked for compliance with the Hardy – Weinberg equilibrium using the Pearson's χ2 test.

Statistical analysis was performed using the Statistica 10.0 software (StatSoft Inc., USA). The Shapiro – Wilk test assessed the normality of the sample distribution. Quantitative data were presented as the median and the interquartile range $Me[Q_1; Q_3]$. The differences between the groups were assessed using the Mann – Whitney *U* test. Qualitative data were presented as frequency of occurrence in absolute values and percentages. For qualitative data, the differences between the groups were determined using the Pearson's χ2 or Fisher's exact tests. A cluster analysis identified homogeneous data. The results were considered statistically significant at $p < 0.05$.

RESULTS

Determination of the SERCA2a and CASQ2 proteins in the patients' myocardium showed that the considered sample is heterogeneous in the expression level of these proteins. Thus, the median SERCA2a level in the total sample was 0.667 [0.334; 1.38], and the median CASQ2 level was 0.506 [0.324; 0.858]. Given the great differences in protein expression among the patients, the method of cluster analysis was used to determine possible homogeneous clusters. It resulted in the identification of two clusters significantly different from each other for each protein under study. According to the expression level of SERCA2a and CASQ2, the total sample of patients was subsequently divided into 2 groups: group 1 – patients with a low protein level; group 2 – patients with a high protein level (Fig. 1).

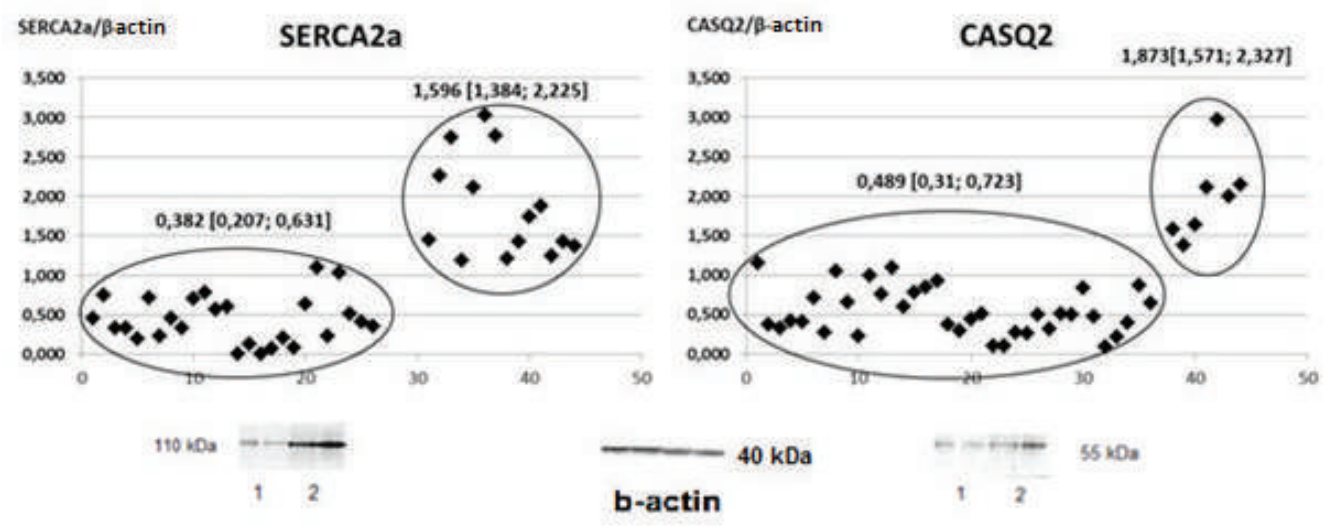

Fig. 1. Clustering of the patients' sample by the expression level of the SERCA2a and CASQ2 proteins

To assess the possible role of the genetic component in the expression of the SERCA2a and CASQ2 proteins in the myocardium of AF patients, we investigated the relationship between the level of these proteins and the presence of polymorphic variants of the *ATP2A2* and *CASQ2* genes. It turned out that all patients were carriers of the polymorphic variant rs1860561 of the *ATP2A2* gene. At the same time, 30 (67%) patients were carriers of the homozygous GG genotype, and 15 (33%) patients were carriers of the heterozygous GA genotype. No patients under study were carriers of the AA genotype.

All patients in the sample were carriers of polymorphic variants rs6684209 and rs7521023 of the *CASQ2* gene. For the polymorphic variant rs6684209, the heterozygous CT genotype was detected in 16 (36%) patients, and the homozygous CC genotype – in 29 (64%) patients. There were no patients with the TT genotype in the study sample. The bulk of the sample with the polymorphic variant rs7521023 (31 patients, 69%) was represented by carriers of the heterozygous genotype AG. 9 (20%) and 5 (11%) patients were carriers of its homozygous genotypes (AA and GG), respectively.

The possible functional significance of each identified genotype was assessed. It turned out (Fig. 2) that in carriers of the homozygous GG genotype of the *ATP2A2* gene, the expression level of the SERCA2a protein was significantly higher (p = 0.039) than in patients with the heterozygous genotype GA and amounted to 0.926 [0.282; 1.65] versus 0.559 [0.123; 1.21], respectively.

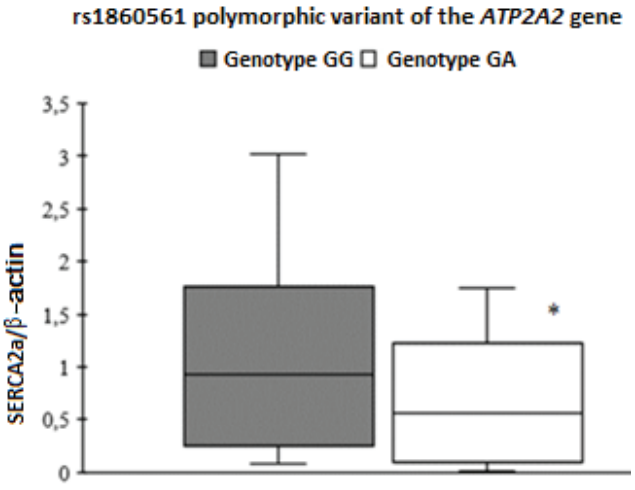

Fig. 2. Expression of the SERCA2a protein depending on the genotypes of the *ATPA2* gene. Here and in Fig. 3: * $p < 0.05$ – statistically significant difference.

The results presented in Fig. 3 show that in carriers of the homozygous CC genotype for the polymorphic variant rs6684209 of the *CASQ2* gene, protein expression was 2.5 times higher than in carriers of the heterozygous genotype and was equal to 0.779 [0.506; 1.380] versus 0.315 [0.272; 0.400], respectively (p = 0.035). The level of protein expression in patients with homozygous genotypes (AA and GG) of the polymorphic variant rs7521023 of the *CASQ2* gene was 0.729 [0.994; 0.517] and 0.516 [2.111; 1.061], respectively, and in patients with the heterozygous genotype AG – 0.479 [0.779; 0.625].

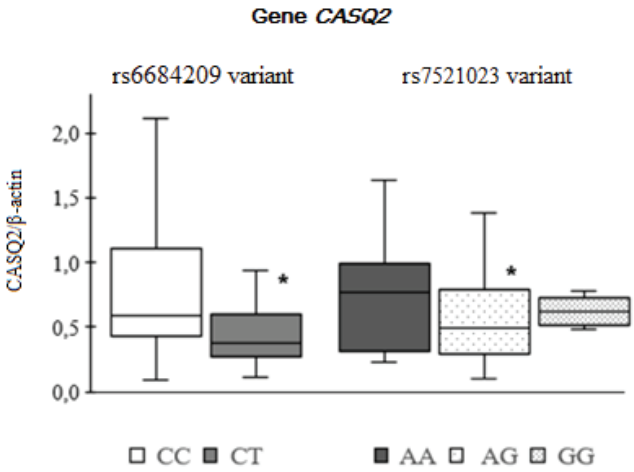

Fig. 3. Expression of the CASQ2 protein depending on the genotypes of the polymorphic variants in the *CASQ2* gene

Analysis of the data on the distribution of patients by clusters depending on the expression level of the SERCA2a protein showed that group 1 with a low level of SERCA2a expression (0.382 [0.207; 0.631]) included 29 patients (64%), and group 2 with a high protein level (1.596 [1.384; 2.225]) consisted of 16 patients (36%). According to the data presented in Table 1, the patients of the first and second groups were comparable in terms of clinical and anamnestic parameters and therapy. However, patients in group 1 were more often prescribed anticoagulants, while patients in group 2 were prescribed antiplatelet drugs.

Table 2 shows the EchoCG results of the patients in the formed groups. Such parameters as the size of the left atrium (LA) and end-systolic and end-diastolic volumes (ESV and EDV) were significantly higher in group 2 than in group 1. At the same time, the rates of early (peak E) and late (peak A) left ventricular diastolic filling were significantly higher in the patients of group 1.

Table 1

Clinical and anamnestic parameters of patients

Parameter	Group 1, $n = 29$	Group 2, $n = 16$	p
Age, years, $Me[Q_1; Q_3]$	45 [40; 51]	42 [38; 48]	0.712
Sex, male / female (n)	19/7	12/2	–
Hypertensive heart disease, n (%)	10 (39)	4 (29)	0.630
Coronary artery disease, n (%)	2 (8)	2 (14)	0.566
Medication			
Statins, n (%)	2 (8)	0	0.300
Antiplatelet agents, n (%)	2 (7)	5 (31)	0.031
Anticoagulants, n (%)	21 (72)	5 (31)	0.008
Antiarrhythmic drugs			
Amiodarone, n (%)	6 (23)	5 (36)	0.544
Allapenin, n (%)	1 (4)	1 (7)	0.902
Propafenone, n (%)	9 (35)	2 (14)	0.269
Sotalol, n (%)	4 (15)	2 (14)	0.915
Bisoprolol, n (%)	3 (12)	0	0.206
Metoprolol, n (%)	2 (8)	2 (14)	0.566

Note: пroup 1 – low level of SERCA2a expression; group 2 – high level of SERCA2a expression.

Table 2

Main structural and functional parameters of the heart in AF patients, *Me* [Q_1; Q_3]

Parameter	Group 1, $n = 29$	Group 2, $n = 16$	p
Ejection fraction, %	65 [62; 68]	63 [60; 66]	0.189
EDV	104 [97; 114]	115 [96; 127]*	0.015
ESV	36 [34; 42]	42 [39; 48]*	0.032
EDD, mm	49 [48; 50]	49.8 [45; 52]	0.902
ESD, mm	31[30; 34]	32 [29; 37]	0.744
LA, mm	37 [35; 42]	42 [39; 45]*	0.035
peak_E, cm / s	82 [72; 88]	69 [62; 80]*	0.039
peak_A, cm / s	61 [59; 66]	47 [44; 52]*	0.018
E / A	1.28 [1.26; 1.5]	1.27 [1.19; 1.55]	0.89
Stroke volume, ml	69.5 [63; 78]	71.5 [59; 79]	0.513
MM, g	174 [157; 186]	173 [138; 211]	0.636
MMI, g / m^2	86.5 [80; 93]	83.5 [71; 95]	0.463

Note: LA – left atrium; ESV – end-systolic volume; EDV – end-diastolic volume; EDD– end-diastolic dimension; ESD – end-systolic dimension; LV – left ventricle; MM – myocardial mass; MMI – myocardial mass index.

A comparative analysis of EchoCG data from patients distributed according to the level of CASQ2 expression in the myocardium did not reveal significant differences between the groups.

DISCUSSION

It is known that the main cause of AF is an abnormal impulse (ectopic activity) [13]. The molecular mechanism of this phenomenon is largely associated with the intracellular homeostasis of calcium ions in cardiomyocytes [14]. Overload of calcium ions in the sarcoplasm of cardiomyocytes leads to a decrease in the electrical stability of the membranes in the cardiac cells and emergence of ectopic pacemakers. Therefore, atrial electrophysiological properties alter, the so-called electrical remodeling occurs [15]. A high atrial rate can stimulate the adaptive response in cardiomyocytes, which is expressed in increased expression of the SERCA2a and CASQ2 proteins.

Thus, the studies by J. Dai et al. found that in AF patients, the expression level of calcium-handling proteins of SR (SERCA2a, phospholamban and ryanodine receptors) in cardiomyocytes was significantly higher than in patients without supraventricular arrhythmias, while the expression level of contractile proteins (troponin T and I, myosin) in the myocardium did not differ [16].

Unfortunately, the available literature does not have any data on the relationship of the polymorphic variants of the *ATP2A2* and *CASQ2* genes with the expression level of their proteins. Our study showed that the presence of specific polymorphic variants and genotypes of the genes of the studied proteins is also significant. It turned out that in carriers of the homozygous GG genotype of the rs1860561 variant of the *ATP2A2* gene and the CC genotype of the rs6684209 variant of the *CASQ2* gene, the level of SERCA2a and CASQ2 expression was significantly higher than in patients with the heterozygous genotype of these genes.

Electrical remodeling can be accompanied by structural changes in the myocardium. In this study, higher SERCA2a expression was associated with greater EDV and ESV values, although these parameters were within the reference values. In this regard, higher EDV values (within the reference values) can be considered as evidence of better maintenance of ventricular diastolic function. This interpretation is quite consistent with the data that SERCA2a overexpression in rabbits with induced AF is accompanied by an increase in the duration of the effective refractory period and an improvement in the myocardial structure [8]. It is possible that excessive hemodynamic load on the atrium in AF adaptively leads to an increase in SERCA2a expression.

This assumption is consistent with the results of our studies, which showed that patients with elevated SERCA2a levels have increased LA size. It is well known that the ventricular filling is divided into two phases: the phase of rapid (active) filling, which is early diastole, and the phase of slow (passive) filling,

corresponding to late diastole, which ends with atrial systole. The phase of rapid filling of the LV characterizes an active relaxation process. At the cellular level at this time, the acto – myosin cross-bridges are disconnected, which is followed by the release of calcium ions into the myoplasm and their reuptake into the SR [17].

Our study showed that patients with a high level of SERCA2a expression had a lower rapid filling rate in early diastole. In addition, these patients had a lower slow filling rate in the late diastole than those with lower SERCA2a expression. The study failed to identify associations of EchoCG parameters of the patients' heart with the level of CASQ2 expression, which may be due to the small number of patients with low CASQ2 expression in our sample.

CONCLUSION

The results of the study allow to conclude that the genotypes of the rs1860561 variants of the *ATP2A2* gene and rs6684209 of the *CASQ2* gene can determine the expression level of SERCA2a and CASQ2. The expression level of SERCA2a is associated with the structural and functional parameters of the heart in AF patients. The obtained results confirm that it is promising to assess the expression level of SERCA2a and CASQ2 to predict the course of cardiovascular pathology and select individual treatment.

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Kondratieva D.S. – determine of the target proteins expression, analyzing and interpreting the data, writing the text of the article. Afanasiev S.A. – develop of the article concept and design, substantiation of the manuscript. Muslimova E.F. – collection of material, determine of the polymorphism of the target genes, data analysis. Archakov E.A. – selection and management of patients, analysis and interpretation of clinical data. Batalov R.E. – analysis of clinical data, verification of critical intellectual content.

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