Abstract—According to the modern concepts, alterations of apoptosis and its genetic regulation are associated with the etiopathogenesis of schizophrenia, which is observed at both the brain and peripheral blood levels. However, studies of this phenomenon are at the initial stage, and the molecular and cellular mechanisms that underlie the anomalies of the processes of apoptotic cell death in schizophrenia are unclear. In the present study, we determined the levels of apoptotic markers, annexin A5 and H-ficolin proteins, in the sera of patients with chronic and first-episode schizophrenia and healthy subjects to test the proposed relationship between schizophrenia and the rs11575945 (−1C/T) single-nucleotide substitution (functional polymorphism) of Kozak consensus sequence in the regulatory region of the annexin A5 gene. Methods of a solid-phase enzyme-linked immunosorbent assay and polymerase chain reaction with allele-specific primers were used. It was shown that the pathogenesis of schizophrenia is characterized by an increased rate of apoptosis, which is more pronounced in the case of the first-episode neuroleptic-free patients than in the case of chronic patients that receive typical neuroleptic haloperidol. It was also shown that the rs11575945 polymorphism of the annexin A5 gene is associated with schizophrenia, and its minor allele is responsible for higher levels of the annexin A5 protein in the blood and represents one of the risk factors for the development of this disease.

**Abbreviations**: OR, odds ratio; CI, confidence interval.

**Keywords**: annexin A5, apoptosis, polymorphism rs11575945 of the annexin A5 gene, H-ficolin, schizophrenia

Abnormal apoptosis is a pathological feature of many diseases [1] including psychiatric [2]. One of the contemporary hypotheses of etiology and pathogenesis of schizophrenia (“neurodevelopmental hypothesis”) proposes that apoptotic processes and their genetic regulation are altered at an early stages of the brain development as well as in mature organism of the patients [3]. It is proposed that both pre- and postnatal as well as genetically determined abnormalities of the apoptotic processes might be among factors responsible for the development of schizophrenia [4] and define the defects of synaptic plasticity [5], neurodegenerative alterations [6], and immune system dysfunction [7–10] peculiar for this pathology. This dysfunction is manifested at both the central nervous system (CNS) and systemic levels; in particular, it is characterized by development of autoimmune and inflammatory reactions [7–10] directly linked to apoptosis [11, 12]. Alterations in the apoptotic processes in schizophrenia are detected at the levels of the brain [13–16] and peripheral blood [17]. However, all of these studies are at the initial stage, and the molecular and cellular mechanisms underlying anomalies during apoptotic cells death in schizophrenia are unclear.

In recent years, in studies of apoptotic alterations and their interrelation with alterations of the immune response, attention is focused on the annexin A5 protein. This protein is able to bind to negatively charged phospholipids, which includes phosphatidylyserine, which already transfers from the inner to the outer membrane of the cell undergoing apoptosis. The membrane-bound form of annexin A5 is an important modulator of the process of phagocytosis of apoptotic cells and inflammatory reactions directed to the removal of dying cells. Increased levels of this protein induce development of autoimmune and inflammatory reactions [18–21]. It was shown that annexin A5 coupled to membrane of apoptotic cells is a ligand for C1q protein [22], which initiates the complement classical cascade, which is hyperactivated in schizophrenia [23]. The soluble form of annexin A5 is considered to be a marker of apoptosis; its sources are apoptotic cells and their fragments. Increased blood levels of soluble annexin A5 suggest the hyperfunction (accelerated rate) of apoptosis and, on the contrary, the low content of this protein in the blood, compared to the norm, suggests the hypofunction (decelerated rate) of apoptosis [18–21].

Data related to the annexin A5 levels in schizophrenia are only presented in one report [24], where the increased serum levels of this protein were found in a small cohort of patients (39 individuals) treated with atypical neuroleptics.

Ficolins are multivalent serum proteins that recognize microbial and apoptotic cells. Disturbances at the level of the gene expression of these proteins are
detected in the pathogenesis of many diseases, in particular in those characterized by dysfunction of apoptosis [25]. H-ficolin is able to bind to a surface of apoptotic cells, activate complement by lectin pathway, and act as an opsonin [26]. It was previously shown that hyperactivation of the complement lectin pathway is also characteristic for patients with schizophrenia [23]. However, to date, there is no literature data relative to H-ficolin in this pathology.

In the present work, the levels of annexin A5 and H-ficolin were determined in the sera of chronic schizophrenia patients treated with typical neuroleptic haloperidol compared to first-episode not treated with neuroleptics patients and healthy subjects. The results of the correlation analysis of the data are presented. It was also assessed whether there is an association between schizophrenia and the (–1C/T) single nucleotide functional substitution (polymorphism rs11575945) in the Kozak consensus sequence of the regulatory part of the annexin A5 gene, which plays a key role in the initiation of transcription [27]. The relation between the annexin A5 blood levels and genotypes of the rs11575945 polymorphism was evaluated. A small part of this work related to annexin A5 content and obtained upon investigating a less number of study subjects was earlier published [28].

**EXPERIMENTAL**

**Study subjects** were patients with the paranoid form of schizophrenia diagnosed by psychiatrists at the Psychiatric Medical Center of the Ministry of Health of the Republic of Armenia based on the criteria of the International Classification of Diseases (ICD-10 code: F20.0). The control group of physically and mentally healthy individuals was composed from donors of the Erebouni Medical Center of the Ministry of Health of the Republic of Armenia. In total, 225 chronic patients treated with typical neuroleptic haloperidol, 25 first-episode neuroleptic-free patients, and 225 healthy subjects (control group) were investigated. In the group of chronic patients 154 males and 71 females were enrolled. The mean age (M ± δ) in this group was 44.2 ± 9.8 years, mean duration of disease—19.5 ± 7.2 years, and age at the first-onset of disease—26.4 ± 8.3 years. In the second group of the first-episode patients 17 males and 8 females were involved, mean age—26.4 ± 9.2 years, mean duration of disease—26.4 ± 9.2 years. In the control group 154 males and 71 females were involved, mean age—42.6 ± 9.2 years. Ninety four chronic and 10 first-episode patients had hereditary predisposition to this disease (family history). One hundred sixteen chronic and 18 first-episode patients with schizophrenia as well as 146 healthy subjects were nicotine-dependent (smoking of tobacco cigarettes). Healthy subjects passed preliminary psychiatric examination, which proved the absence of personal or family history of any psychiatric illness. The presence/absence of family history of schizophrenia or any other psychiatric illness was assessed on the base of interview, history of disease, as well as information available in the databases of the Psychiatric Medical Centre of the Ministry of Health of the Republic of Armenia. All subjects did not take any medication for at least one month before study. None of them suffered from neurological, endocrine, acute or chronic infectious, autoimmune, autoinflammatory, oncological or other serious diseases and was subjected to surgical intervention for at least 12 months before blood sampling. All study subjects were ethnic Armenians living in the territory of Armenia.

All subjects were informed about the study by physicians and gave their consent to blood collection. The study was approved by the Ethical Committee of the Institute of Molecular Biology of the National Academy of Sciences of the Republic of Armenia (international registration number 00004079).

Fasting blood was collected from cubital vein and kept cool on ice. Part of the blood was placed in tubes without coagulant and used to obtain serum, another part—in tubes containing EDTA as anticoagulant. To obtain plasma, blood was centrifuged at 3000 g for 10 min, and the supernatant was collected. DNA from the blood was isolated using the standard phenol-chloroform method [29]. Serum and DNA samples were stored at −30°C. All samples were analyzed in duplicates.

Concentrations of annexin A5 and H-ficolin in the blood serum were measured using a solid-phase enzyme-linked immunosorbent assay (ELISA) and commercial kits (Uscn Life Science Inc., United States and Hycult Biotech Inc., Netherlands, respectively) according to the manufacturers’ instructions.

The rs11575945 polymorphism of the annexin A5 gene was evaluated by genotyping DNA samples of chronic patients and healthy subjects using the polymerase chain reaction with allele-specific primers (PCR-SSP) [30]. The oligonucleotide primers were designed using database of the International Center of Biotechnology (GenBank, www.ncbi.nlm.nih.gov, GeneID:308). The following primers were designed and used: C-allele-specific CCGACCCGAGTAGTCGCC; T-allele-specific CCGACCCGAGTAGTGC; and constant primer GCCACGTCACAACGCTTGTC. As control primers, we used the TGCCAAGTGAGCACCACA and GCATCTGTGTCTGAGAT sequences. To check a reproducibility of the data, randomly selected samples (10% of total) were genotyped twice, and it was shown that repeats of all randomly selected samples were identical. The electrophoresis of the products of DNA amplification was performed in 2% agarose gel using 0.045 M Tris-borate buffer, pH 8.0, which contains 0.001 M EDTA and ethidium bromide (0.7 μg/mL). The bands in the gel were visualized using a contact UV lamp.