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Original Scientific Paper

EXPRESSION OF CONNEXIN-40 AND INTERLEUKIN 6 GENES IN PATIENTS WITH ATRIAL FIBRILLATION

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SUMMARY – Atrial fibrillation (AF) is the outcome of the rapid onset of abnormal electrical impulses in the atria. These impulses prevent the heart natural pacemaker, which normally controls the heart beat, from doing its job. The heart rate becomes highly asymmetric. The purpose of this case-control study was to determine the role of the connexin-40 (*Cx-40*) and interleukin-6 (*IL-6*) gene expression in patients suffering from AF in Pakistan. There were 200 subjects included in this study. The subjects were categorized into two groups as control and AF groups (n=100 each). RNA separated from blood was used for analysis of the *Cx-40* and *IL-6* gene expression by real-time polymerase chain reaction. The *Cx-40* gene expression profile was increased 10.2-fold in AF subjects as compared to 1.9-fold in control group. The *IL-6* gene expression profile was decreased 1.5-fold in AF group as compared to 6.1-fold in control group. Results of the study suggest that development of AF in the Pakistani population was linked to the increased expression of *Cx-40* gene and lower expression of *IL-6* gene.

Key words: *Atrial fibrillation; Connexin-40; Interleukin-6; Gene expression*

Introduction

Atrial fibrillation (AF) is an extremely prevalent worldwide arrhythmia of the heart and continues to be a major factor in morbidity and mortality[1]. The incidence of AF, as well as the prevalence of the disease is rising at the global level[2]. Over the last three decades, there has been a significant rise in the prevalence of AF, which is now estimated to affect 60 million people worldwide[3]. Chronic types of AF lead to the risks of thromboembolism and cardiac failure[4,5]. Nevertheless, in some cases no risk factors are identified in AF patients, which demonstrates the possible role of genetics in this disease. Consequently, both genetic and nongenetic factors are responsible for AF[6]. A great deal of research on the genetics of AF has been done due to the limitations of the existing treatments for this condition[7]. Rare variations in ion channels, transcription factors, and many other genetic loci are the source of AF[8]. Gap-junctions (GJs) are intercellular channels mediating electrical coupling between 2 adjacent cardiomyocytes, each one contributing to the junction with a hemichannel or connexins (Cxs), formed by 6 transmembrane proteins or connexins[9,10]. In the human heart, 5 major types of connexins (Cx-37, Cx-40, Cx-43, Cx-45 and Cx-46) have been identified to express till now[11]. A substantial connexin, Cx-40, is

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mainly confined to the tissues of atria, as well as in the conduction system of the ventricles[12,13].

Connexins are a class of highly dynamic proteins involved in conduction in the heart. Generally, there are discrepancies about the precise pattern of alterations in connexin expression when AF is present. Gene mutations in Cx40 and Cx43, although less prevalent, have been identified as the sole cause of AF in both human and animal models. Crucially, research on AF in humans and animals consistently demonstrates that connexin phosphorylation is changed, connexins become lateralized, and connexins are produced differently. There is evidence linking altered connexin phosphorylation and localization to reduced atrial conduction. A reentry-friendly substrate is produced by heterogeneous and delayed atrial conduction. When combined, connexin remodeling plays a major role in maintaining persistent AF. Connexins are essential for the onset and maintenance of AF, as demonstrated by the success of several gene therapies, small peptides, and drug therapies that target connexin remodeling. These interventions have been shown to counteract connexin dephosphorylation and down-regulation in AF, improving cardioversion and lowering AF vulnerability[14]. Another study revealed reduction in the expression of *Cx-40* after variations in the promoter region and onset of AF[15].

A pleiotropic cytokine called interleukin (IL)-6 is generated in response to infections and tissue injuries. Cytokine IL-6 is produced by a variety of cell types, notably fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, mast cells, macrophages, dendritic cells, and T and B cells[16]. Host defense, control, proliferation and differentiation of immune cells are the key physiological processes regulated by IL-6[16].

Recent developments in the field of cardiology have enabled identification of numerous cellular and molecular processes that point to inflammation as the cause of AF. Angiotensin II promotes immune cell recruitment and generation of proinflammatory cytokines such as IL-6, IL-8, and tumor necrosis factor-α (TNF-α) in response to inflammation[10]. Several studies have shown that cardiac inflammation is a significant risk factor for AF in the general population, as well as in people who have had surgical treatment of the heart[17-19]. Increased levels of IL-6 suppress cardiac connexin (Cx-40), which in turn leads to the electrical remodeling of atria[19].

The purpose of this study was to demonstrate the expression profile of two genes, the *Cx-40* gene and *IL-6* gene, in AF patients.

Materials and Methods

The case-control study was performed on AF patients at the Punjab Institute of Cardiology (PIC), Lahore, Pakistan, from April to September 2020. The study was approved by the PIC Review Board (Ref. No: RTPGME-Research-177) and Ethics Review Committee of the Department of Zoology, Lahore College for Women University (LCWU), Lahore, Pakistan. All patients were recruited after receiving their approval to participate in the study. The Rao program was used to calculate the sample size for this investigation, accounting for a 5% margin of error and the disease prevalence. A total of 200 subjects were enrolled, divided into two groups of 100 AF subjects and 100 control subjects. After examining electrocardiograms of the study participants, PIC physicians diagnosed AF based on the uneven R-R intervals, chaotic electrical activity in the place of P waves, and abnormal impulse conduction to the ventricles.

Age (≥18 years) and sex (male and female) were the inclusion criteria. AF patients with a history of stroke, coronary artery disease, myocardial infarction, transient ischemic attack, prior coronary artery bypass graft surgery, systemic embolism, or percutaneous coronary intervention were enrolled in the AF group. AF subjects suffering from other diseases such as renal impairment, anemia and hemorrhage in the last six months, and pregnant women were excluded. Subjects with no history of AF or heart disease were included in the control group.

A questionnaire was distributed to study participants to gather relevant data. Respondents were asked to provide answers to a series of questions presented to them. The questionnaire was designed to elicit comprehensive responses, allowing for thorough understanding of the subject matter under investigation. A questionnaire was designed to ask various questions from patients regarding age (years), sex (male and female), blood pressure, body weight, hypertension, diabetes status, history of AF, and smoking. The questionnaire was filled out by each participant. After collecting responses from the participants, data were

meticulously analyzed to identify trends, patterns, and insights. The questionnaire proved to be an effective tool for gathering valuable information, contributing significantly to the research objectives.

Blood sample collection

The median cubital vein was used to draw blood samples. The blood samples obtained were moved into sterile tubes coated with EDTA to separate RNA and into tubes designated for collecting serum. Serum was extracted after 30 minutes by centrifuging the serum separation container for 15 minutes at 3000 rpm.

Assessment of biochemical parameters

Fasting blood glucose (FBG) was measured by a glucometer. Cardiac markers including cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), and creatine phosphokinase (CPK) were measured on a Biotek Elx800 Microplate Reader by commercially available ELISA kits. The reference range for cTnI was 0-0.04 ng/mL, for CK-MB less than 25 U/L, and for CPK less than 171 U/L.

Extraction of RNA and cDNA synthesis

The RNA was extracted from the blood by using the Trizol method (Refrigerated Centrifuge Machine HARRIER 18/80, UK). Quantification of RNA was performed by Nanodrop (Multiskan SkyHigh Microplate spectrophotometer, UK). Utilizing the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific), mRNA was converted into cDNA for gene expression (Programmable Thermal Cycler Ptc-06 UK) (Thermo Scientific cDNA Kit, Cat #K1622). Gel electrophoresis was performed to confirm the formation of cDNA.

Expression analysis by real-time PCR

Real-time polymerase chain reaction (RT-PCR) was used to confirm the expression profile of both genes that were expressed differently. To design the primers, the bioinformatics tool NCBI Primer 3 was used. RT-PCR was performed using Maxima SYBER Green and adding specific primers of both genes to confirm their expression. The *GAPDH* gene was utilized as interior control standardization (Table 1).

Table 1. Designed primers used in the study

Gene	Primer	Product size (bp)	Tm (°C)
GAPDH *Cx-40*	F: ATC CCA TCA CCA TCT TCC AGGA R: CAA ATG ACC CAG CCT TCT F: CCGGCCCACAGAGAAGAATGT	122	59 °C 60°C
Cx-40	R: TCTGACCTTGCCTTGCTGCTG	465	
IL-6	F: TCTCCACAAGCGCCTTCGGT	77	61°C
IL-6	R: TGGGGCAGGGAAGGCAGC		

Table 2. Baseline demographics, risk factors and comorbidities in study groups

	Variable	Control group	AF group
Gender, n (%)	Male Female	26 (26) 74 (74)	84 (84) 16 (16)
Smokers, n (%)	No Yes	84 (84) 16 (16)	29 (29) 71 (71)
Hypertensive subjects, n (%)	No Yes	82 (82) 18 (18)	32 (32) 68 (68)
Diabetic subjects, n (%)	No Yes	82 (82) 18 (18)	52 (52) 48 (52)
AF history, n (%)	No Yes	100 (100) -----	44 (44) 56 (56)

AF = atrial fibrillation

Statistical analysis

A statistical software IBM SPSS version 24.0 (SPSS Corp., Chicago, Illinois, USA) was used on data analysis. The level of statistical significance was set at $p<0.05$. Data were expressed as mean ± standard deviation (SD) for continuous variables and as number (percentage) for categorical variables. Those continuous variables that did not conform to normality were logarithmically transformed. Differences between AF group and control group were examined with Student's t-test for normal and Mann-Whitney test for non-normally distributed parameters, and χ^2-test for categorical variables.

The relationship of the *Cx-40* gene and *IL-6* gene with clinical parameters (i.e., age, systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), FBG, cTnI, CK-MB, CPK) was examined by using two-tailed Pearson's correlation analysis. Data on gene expression were presented as an n-fold change, and relative expression of *Cx-40* and *IL-6* genes employing comparative CT value (2-ΔΔ CT).

Results

Demographic characteristics of the study groups are shown in Table 2. Nonsignificant differences were recorded in age and BMI, SBP and DBP between the control and AF groups. There were significant differences in the mean values of cardiac enzymes (CK-MB, cTnI and CPK) between the AF group and control group (Table 3).

Cx-40 and IL-6 gene expression profile

Quantitative expression of the two genes, *IL-6* and *Cx-40* genes, was expressed as an n-fold difference in contrast to the reference gene (*GAPDH*). The expression profile of the *Cx-40* gene was increased 10.2-fold in AF subjects as compared to 1.9-fold in the control group. The expression profile of the *IL-6* gene was increased 6.1-fold in the control group as compared to 1.5-fold in the AF group (Table 3).

The relationship of the *Cx-40* gene and *IL-6* gene with clinical parameters (i.e., age, SBP, DBP, BMI, FBG, cTnI, CK-MB, CPK) is shown in Table 4. There was a significant positive correlation between CK-MB ($r=0.731$, $p=0.016$) and expression of the *IL-6* gene in

Table 3. Clinical parameters of AF in study groups

Variable	Control group (n=100)	AF group (n=100)	p-value
Age (years)	60.7±10.3	61.3±12.3	0.78
BMI (kg/m^2)	29.2±8.5	30.5±9.3	0.97
SBP (mm Hg)	129.2±28.3	130.48±11.2	0.94
DBP (mm Hg)	83.12±17.46	87.64±5.13	0.45
FBG (mg/dL)	118.8±19.9	121.0±5.1	0.56
cTnI (ng/mL)	6.67±3.55	0.90±2.15	0.001
CK-MB (U/L)	19.49±2.38	60.59±83.51	0.001
CPK (U/L)	118.38±29.75	1095.85±4251.11	0.001
Expression of *IL-6* gene (arbitrary units)	6.1-fold	1.5-fold	0.01
Expression of *Cx-40* gene (arbitrary units)	1.9-fold	10.2-fold	0.13

AF = atrial fibrillation; SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index; FBG = fasting blood glucose; cTnI = cardiac troponin I; CK-MB = creatine kinase-MB; CPK = creatine phosphokinase; Cx-40 = connexin-40; IL-6 = interleukin-6

Table 4. Correlation analysis of Cx-40 and IL-6 gene expression profile with clinical parameters of AF in study groups

Clinical parameter	r-value of *Cx40* gene		r-value of *IL-6* gene	
	Control group	AF group	Control group	AF group
Age (years)	-0.479	0.003	-0.059	0.044
SBP (mm Hg)	0.040	0.151	0.165	0.070
DBP (mm Hg)	0.085	0.184	0.044	0.110
FBG (mg/dL)	-0.183	-0.190	-0.042	-0.080
BMI (kg/m^2)	-0.265	0.045	-0.254	-0.260
cTnI (ng/mL)	0.389	-0.111	0.286	-0.197
CK-MB (U/L)	0.077	-0.158	0.731*	0.224
CPK (U/L)	-0.337	-0.080	-0.493	0.240

*Pearson correlation coefficient (r)<0.05; AF = atrial fibrillation; SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index; FBG = fasting blood glucose; cTnI = cardiac troponin I; CK-MB = creatine kinase-MB; CPK = creatine phosphokinase; Cx-40 = connexin-40; IL-6 = interleukin -6

the control group. There was no significant association of the *Cx40* and *IL-6* gene expression with clinical parameters (Table 4).

Discussion

The present study was the first to demonstrate the expression profile of *Cx-40* and *IL-6* genes in Pakistani AF patients. In this study, the focus was on two genes, *Cx-40* and *IL-6*, due to their important role in AF. The purpose of choosing these genes was the lack of data available on these genes in the Pakistani population. Action potentials in the heart can spread quickly due to the low resistance passage provided by the gap junctions (GJs) created by Cx-40 and Cx-43. Sporadic somatic mutations in the *GJA5* gene, which codes for the protein Cx-40, have been found in AF[15]. Structural changes either by mutations and polymorphisms or spatial changes of the gene lead to AF condition. Mutations in the *Cx-40* gene lead to alterations in the expression of the *Cx-40* gene and are associated with both trigger creation from the thoracic veins and increased susceptibility of the myocardium of heart atria to AF[20,21]. In this study, we also examined the role of inflammatory marker IL-6 in AF. A differentially expressed cytokine called IL-6 plays a role in a variety of essential processes, including inflammation, hematopoiesis, bone metabolism, and embryonic development[22]. The size of the left atrium has been linked to IL-6. Elevated IL-6 may cause AF by remodeling the left atrium, as increasing left atrial size is known to be a risk factor for the condition[23].

In the present study, nonsignificant differences were recorded in age and BMI of control and AF groups. Nevertheless, it is reported elsewhere that cardiovascular arrhythmias are more likely to occur with aging due to changes in structures, as well as functions of the heart mechanical system, electrical system, and alterations in the metabolism of the body and energy changes[24]. A previous study showed that a significant increase in the probability of AF recurrence was associated with weight changes[25]. A retrospective Italian investigation on AF individuals recorded similar findings, i.e., those with higher and rising BMI had a greater chance of AF recurrence throughout long-term follow-up[26].

Our study demonstrated significant differences in smoking prevalence between the control and AF groups. The association between smoking and AF was confirmed by Chamberlain *et al*.[27].

A widely recognized risk factor for heart disease are blood pressure (BP) fluctuations. In an extensive population-based study, it was described that a significant increase in the risk of AF was related to considerable BP fluctuation in both SBP and DBP[28]. In the

Pakistani population, significant differences were observed in SBP and DBP of the control and AF groups. A study conducted on healthy middle-aged women reports that BP is a significant independent factor of AF. Women with SBP (130 to 139 mm Hg) or DBP (85 to 89 mm Hg) at the time of AF onset were 28% and 53%, more likely, respectively, to experience an AF event than women having SBP (120 mm Hg) or low DBP (65 mm Hg)[29].

Hypertension that is not under control is the main risk factor for the development of persistent AF. Remodeling of atria caused by uncontrolled hypertension aids in the onset and continuation of AF[30]. A variety of excitation pathways contribute to complicated AF. Despite epidemiologic similarities between AF and hypertension, their coexistence in patients is a typical clinical occurrence[31]. In the population of Pakistan, it was seen that more individuals were hypertensive in the AF group than in the control group.

In the present study, the level of FBG in AF patients was different from the control group, suggesting an association of the glucose level and AF condition. The number of patients suffering from diabetes was also higher in the AF group as compared to the control group. According to a systemic review by Aune *et al*.[31], increase in the blood glucose level is associated with an increased risk of AF of 28% and 20% in people suffering from diabetes and prediabetes, respectively. Even at the prediabetes stage, dysglycemia is linked to general cardiovascular disease.

There are several biomarkers associated with the development and progression of AF, and by conducting more comprehensive screenings, it may be possible to reduce the complications associated with AF. In our study, we only used cTnI, CK-MB and CPK as cardiac markers, which have not been reported before. Highly significant differences in cTnI, CPK and CK-MB were also seen between the AF and control groups in the present study.

In this study, the expression of Cx-40 was higher in AF patients as compared to normal subjects. However, the expression of IL-6 was lower in AF patients in comparison to controls. Higher expression of the *Cx-40* gene has a role in AF patients. Our findings are parallel to a study conducted earlier on the role of the *Cx-40* gene in AF[32]. According to previous research, aberrant activation of atria may be connected with the increased expression and disorganized distribution of Cx-40 and Cx-43 proteins in the atria of AF patients[32]. In another study, connexin proteins and mRNAs were quantified by immunoblotting and quantitative RT-PCR[33]. To check for polymorphisms or mutations in the connexin gene, the genomic DNA from PCR-amplified samples was sequenced. According to immunoblotting, Cx-40 protein levels in samples from individuals with paroxysmal and chronic AF were reduced to 77% or 49% of control levels, respectively[33].

The debate about the role of inflammation as a cause or consequence of AF is still unknown. However, both processes are associated[34]. A study found that short-term hypertension enhanced inflammatory cell infiltrates in the atria and led to AF condition, suggesting that inflammation may be the root cause of AF[35]. Higher levels of IL-6 and high-sensitivity C-reactive protein (hsCRP) were found in individuals with AF as compared to healthy subjects, as well as in permanent AF as compared to those with persistent AF[36]. The pathophysiological features of IL-6 in AF, including altered cardiac extracellular matrix and cardiac dysfunction, were documented in a different investigation. They influence cardiac myocyte and fibroblast cell-to-cell interactions, myocardial damage development, and the degree of left ventricular dysfunction, while increasing left atrial size and rapidly inducing atrial electrical remodeling by down-regulating cardiac connexins. Circumstances relating to left ventricular assist devices and atrial electrical remodeling are quickly induced[37]. In a population of Pakistan, the IL-6 profile was lower in the AF group as compared to the control group. Another study evaluating the contribution of inflammatory processes to AF and how inflammatory indicators affect prognosis in terms of predicting long-term risk of AF recurrence following electrical cardioversion showed that cardioversion had an initial success rate of 88% and a total recurrence rate of 68% after 180 days. The levels of hs-CRP and IL-6 were considerably higher in patients with permanent AF. Lower levels of IL-6 in AF patients might be due to the utilization of medicines during their stay in the hospital[38,39].

Moreover, this study did not find serum concentrations of IL-6 and Cx-40 in AF patients in the Pakistani population. A limitation of this study is that the exact mechanism of action of IL-6 and Cx-40 is not fully understood.

Conclusion

It is concluded that the increased expression of *Cx-40* and lower expression of *IL-6* genes might have a significant role in the development of AF in the Pakistani population. It was evaluated that the alterations in *Cx-40* and *IL-6* genes were the possible genetic factors for AF. However, the specific mechanism of the gene involvement in AF pathogenesis has not yet been determined. There was no significant association of the expression of *Cx40* and *IL-6* genes with clinical parameters. By utilizing this information, clinicians may be able to provide patients with more individualized illness management programs. Additional substantial prospective investigations are required to confirm our findings.

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Sažetak

EKSPRESIJA GENA KONEKSINA-40 I INTERLEUKINA-6 KOD BOLESNIKA S ATRIJSKOM FIBRILACIJOM

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Atrijska fibrilacija (AF) je rezultat brzog početka abnormalnih električnih impulsa u pretkomorama. Ovi impulsi sprječavaju prirodni pejsmejker srca koji normalno kontrolira srčani ritam da obavlja svoj posao. Srčana frekvencija postaje veoma asimetrična. Cilj ove studije slučaja i kontrola bio je utvrditi ulogu ekspresije gena koneksin-40 (*Cx-40*) i interleukin-6 (IL-6) kod bolesnika s AF u Pakistanu. U ovu studiju je uključeno 200 ispitanika. Ispitanici su podijeljeni u dvije skupine, kontrolnu i AF (n=100 svaka). RNK izdvojena iz krvi je korištena za analizu ekspresije gena *Cx-40* i *IL-6* pomoću lančane reakcije polimerazom u stvarnom vremenu. Profil ekspresije gena *Cx-40* bio je povećan 10,2 puta kod ispitanika s AF u uporedbi s 1,9 puta u kontrolnoj skupini. Profil ekspresije gena *IL-6* je smanjen 1,5 puta u skupini s AF u usporedbi sa 6,1 puta u kontrolnoj skupini. Rezultati studije ukazuju na to da je razvoj AF kod pakistanske populacije povezan s povećanom ekspresijom gena *Cx-40* i nižom ekspresijom gena *IL-6*.

Ključne riječi: *Atrijska fibrilacija; Koneksin-40; Interleukin-6; Ekspresija gena*