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Research Article

Analysis of *FOXO4* Gene Polymorphism in Cardiac Patients from Faisalabad, Pakistan

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ABSTRACT
Background: Cardiovascular disease (CVD) involves complex interactions between genetic susceptibility and metabolic risk factors. Forkhead box O4 (*FOXO4*) regulates oxidative stress, inflammation, and cellular survival. This study investigated two *FOXO4* polymorphisms and their associations with CVD and selected biochemical parameters in adults from Faisalabad, Pakistan.
Methods: This case–control study included 100 patients with clinically diagnosed CVD and 50 apparently healthy controls aged over 30 years. Venous blood samples were collected for molecular and biochemical investigations. Genotyping targeted rs1404536455 (G/A) and rs1222386114 (T/C). Biochemical assessments included serum lipid profiles, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, and uric acid. Genotype distributions were compared using an exact test, and biochemical comparisons were evaluated using one-way analysis of variance.
Results: For rs1404536455, the AA genotype occurred in 24.0% of patients and 8.5% of genotyped controls, with a significant difference in overall genotype distribution ($p = 0.010$). No significant genotype-distribution difference was observed for rs1222386114 ($p = 0.409$). Unadjusted biochemical comparisons identified differences across rs1404536455 genotypes in ALT ($p = 0.019$) and creatinine ($p = 0.048$), and across rs1222386114 genotypes in LDL cholesterol ($p = 0.007$), ALT ($p = 0.004$), and uric acid ($p = 0.006$). Other biochemical comparisons were not statistically significant.
Conclusion: *FOXO4* rs1404536455 demonstrated differences in genotype distribution between cardiovascular patients and healthy controls, while selected biochemical parameters varied across genotype groups. These findings provide population-specific insights into the potential contribution of *FOXO4* genetic variability to cardiovascular disease.

Keywords: Cardiovascular Disease, *FOXO4*, Gene Polymorphism, Genetic Susceptibility, Lipid Profile, Oxidative Stress.

INTRODUCTION

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality worldwide, imposing a substantial burden on healthcare systems, particularly in low- and middle-income countries[1]. Cardiovascular disorders develop through a complex interplay of genetic risk, metabolic disorders, environmental exposures and lifestyles. The rising incidence in Pakistan of coronary artery disease, myocardial infarction, hypertension and other cardiovascular complications has emphasized the need to understand the molecular determinants of disease susceptibility and progression. While the conventional risk factors such as dyslipidaemia, diabetes mellitus, obesity, smoking and hypertension are well accepted, they are insufficient to account for interindividual variation in susceptibility to cardiovascular disease. Therefore, the study of genetic polymorphisms related to vascular homeostasis, oxidative stress control and inflammatory signaling could offer further information on cardiovascular pathophysiology[2,3].

Oxidative stress and inflammation are basic mechanisms that can lead to the development and progression of cardiovascular diseases[4]. Overproduction of reactive oxygen species (ROS) leads to lipid peroxidation, protein oxidation, mitochondrial dysfunction and DNA damage, disrupting cellular redox homeostasis. These molecular changes affect the endothelial function and proliferation of vascular smooth muscle cells and lead to cardiomyocyte injury and adverse cardiac remodeling. Mitochondrial ROS production and activation of stress-responsive signaling pathways promotes

cardiomyocyte apoptosis, myocardial fibrosis and progressive ventricular dysfunction during myocardial ischemia–reperfusion injury[5]. In addition, inflammatory molecules such as cytokines, chemokines and activated leukocytes lead to the activation of the endothelium, the degradation of the extracellular matrix and the formation of atherosclerotic plaques. Thus, the interplay of oxidative stress and inflammatory signalling is a key factor in the determination of cardiovascular injury and subsequent tissue remodelling[6].

Genetic determinants of cardiovascular vulnerability include modulation of cellular responses to oxidative damage, inflammation, apoptosis and metabolic abnormalities. The forkhead box O (FOXO) family of transcription factors has been highlighted for their contribution to cellular homeostasis and orchestration of stress adaptive responses among the transcriptional regulators involved in these processes[7,8]. The mammalian FOXO family comprises four major transcription factors, FOXO1, FOXO3, *FOXO4*, and FOXO6, which regulate genes associated with antioxidant defense, cell-cycle regulation, apoptosis, autophagy, DNA repair, and energy metabolism. Their transcriptional activities are regulated by intracellular signaling pathways, especially the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, which controls FOXO phosphorylation, nuclear localization and interactions with downstream transcriptional targets. Impaired regulation of these pathways can result in changes of cellular resistance to metabolic and oxidative stress, and may play a role in pathological changes in cardiovascular tissues[9,10].

Forkhead box O4 (*FOXO4*) is a key member of the Forkhead box O (FOXO) family of transcription factors that play a role in cellular differentiation, regulation of inflammation, response to oxidative stress, and vascular homeostasis[11]. *FOXO4* activity is regulated by post-translational modifications such as phosphorylation, acetylation and ubiquitination that affect its stability, cellular localization and transcriptional activity[12]. Experimental studies have shown the involvement of *FOXO4* in endothelial inflammatory responses and its role in vascular injury mechanisms. *FOXO4* in particular, has been shown to regulate endothelial arginase-1, suggesting its role in early inflammation after myocardial infarction (MI), a condition involving myocardial injury and repair. In addition, there is experimental evidence that *FOXO4* could affect cardiomyocyte survival in ischemia–reperfusion injury via pathways that include oxidative stress and apoptosis. All these observations suggest that the cardiovascular phenotype of *FOXO4* is context dependent and could vary depending on the tissue properties, pathological processes, and cellular context[13].

FOXO4 is also associated with cardiovascular disease, which is important for metabolic control and vascular dysfunction. Dyslipidaemias, such as increased levels of low-density lipoprotein cholesterol, hypertriglyceridaemia and impaired cholesterol homeostasis, are all involved in the development of atherosclerotic plaques and cardiovascular complications[14]. Hepatic enzyme abnormalities and renal dysfunction are also commonly seen in association with cardiovascular disease, and may be secondary to metabolic abnormalities or systemic disease. The biochemical characteristics might be affected by transcriptional pathways that affect the adaptation to oxidative stress and metabolic homeostasis[15]. So far, however, associations of particular *FOXO4* genetic polymorphisms with commonly measured biochemical parameters of lipids, liver and kidneys are not well described. Exploring these associations might help explain if there is a biochemical difference among individuals with cardiovascular disease that could be linked to *FOXO4* polymorphisms[16].

Single-nucleotide polymorphisms (SNPs) are an important source of human genetic variability that can affect disease susceptibility via gene expression, transcriptional regulation or change protein function[17]. Variations in these transcription factors involved in oxidative stress and inflammatory pathways may play a role in the susceptibility to cardiovascular disease. The functional roles of FOXO proteins have been well studied, but the clinical relevance of the individual *FOXO4* polymorphisms to cardiovascular disease has not yet been fully understood. Furthermore, the prevalence of and effects of genetic variants can vary between groups due to genetic ancestry, environmental exposure, and cardiovascular risk factors. It is, therefore, advantageous to conduct studies on a population-specific level to get insights into the association between *FOXO4* genetic variability and cardiovascular disease, especially in Pakistani population where lacks of adequate data on such associations[18].

This study aimed at assessing the relationship of polymorphisms in *FOXO4* gene (rs1404536455 G/A, rs1222386114 T/C) with

cardiovascular disease (CVD) in the population of Faisalabad, Pakistan. Further, the association of these polymorphisms with biochemical parameters such as total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, ALT, AST, urea, creatinine and uric acid was assessed. The objective of the study was to describe the distribution of the selected *FOXO4* polymorphisms in cardiovascular patients and controls and to investigate the link between *FOXO4* polymorphisms and lipid metabolism, hepatic enzyme activity, and renal function, using molecular genetic analysis combined with biochemical profiling.

MATERIALS AND METHODS

Study Design and Setting

The study was an analytical case–control study conducted at the Diabetes and Cardio-Metabolic Disorders Laboratory, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. Clinical recruitment and biological samples collection were performed at Allied Hospital, Faisalabad, and Faisalabad Institute of Cardiology, Faisalabad, Pakistan. The study aimed to examine the association of forkhead box O4 (*FOXO4*) gene polymorphisms with cardiovascular disease (CVD) and explore the association of these gene polymorphisms with lipid profile, hepatic enzyme activity, and parameters of renal function. Clinical data, biochemical parameters and molecular genetic data were collected and analysed to assess the distribution of the genetic variants studied and their association with cardiovascular disease.

Study Population and Participant Selection

A total of 150 subjects, 100 with clinically diagnosed cardiovascular disease and 50 apparently healthy controls, above 30 years old, of both sexes, were included in the study. Patients were selected from the departments of Medicine and Coronary Care Unit 1 & 2 of Allied Hospital Faisalabad and Faisalabad Institute of Cardiology. Cardiovascular disease was diagnosed by clinical assessment, confirmed by electrocardiographic findings and exercise tolerance test and confirmed by consultant cardiologist. The healthy controls were selected from the residential areas of Faisalabad and NIBGE Campus. The control group was made up of people with no known chronic infectious or noninfectious diseases, malignancy, significant organ pathology or persistent infections. Clinical histories and biological specimens were obtained from the study participants for biochemical and molecular investigations.

Demographic, Anthropometric, and Clinical Assessment

Demographic and clinical characteristics were recorded, through a structured questionnaire. Data on age, sex, family history of disease, dietary habits, physical activity, use of medication, blood pressure, and body mass index (BMI) was obtained. To characterize the disease status among patients, clinical records were assessed and appropriate cardiovascular investigations were performed. Laboratory parameters data were obtained from lipid profile, liver enzymes, kidney function tests, and other clinical chemistry and haematological results that were available. These data were included in biochemical and genetic analyses.

Blood Sample Collection and Processing

A total of approximately 6 mL of peripheral venous blood was drawn from each participant in an aseptic manner. The collected blood was split into two parts, whereof 3 mL was stored in an ethylenediaminetetraacetic acid (EDTA) containing vacutainer for isolation of genomic DNA and the remaining 3 mL was stored in a gel-coated serum separation tube for biochemical analyses. Serum was left to coagulate at room temperature for about 60 minutes and then centrifuged at 3500 rpm for 5 minutes. The serum was separated and transferred carefully into sterile 1.5 mL microcentrifuge tubes for biochemical analysis which were kept at −20°C. EDTA-anticoagulated blood samples were used for subsequent molecular genetic investigations.

Biochemical Analysis

Biochemical parameters assessed were serum total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine and uric acid. Commercially available diagnostic reagent kits were used for the documented lipid and hepatic enzyme assays. Spectrophotometric measurements were made using the BioSystems BTS-330 biochemical analyzer using the procedures outlined for each assay.

Assessment of Serum Lipid Profile

An enzymatic colorimetric method from DiaSys Diagnostics was used to measure serum total cholesterol. The principle employed was the

enzymatic hydrolysis of cholesterol esters, oxidation of cholesterol and determination of hydrogen peroxide. This resulted in the production of hydrogen peroxide which reacted with 4-aminoantipyrine and phenol in the presence of peroxidase to get a coloured quinoneimine complex. After incubating at room temperature for about 20 minutes, the absorbance was taken at 546nm. The concentrations of total cholesterol were determined in comparison to the corresponding standard and the results were presented in mg/dL.

Triglyceride levels in serum were measured with Crescent Diagnostics' commercial enzymatic colorimetric reagent kit. Lipoprotein lipase was used to break down the triglycerides into glycerol and free fatty acids. Enzymatic reactions with glycerol kinase and glycerol phosphate oxidase produced hydrogen peroxide, which then was involved in the colorimetric reaction with a peroxidase. The reaction mixtures were allowed to incubate at 37°C for about 8-10 minutes and absorbance was measured at 546 nm. The triglycerides concentration was determined using a standard provided and reported in mg/dL.

High density lipoprotein cholesterol was measured by precipitation method by Crescent Laboratories commercial reagent kit (United Kingdom). Chylomicrons, very-low-density lipoproteins and low-density lipoproteins were precipitated with phosphotungstic acid in the presence of magnesium ions. After separation, the amount of cholesterol in the supernatant was measured spectrophotometrically at 546 nm. The LDL-C was determined by subtracting HDL-C and VLDL-C from the total cholesterol. The lipids were measured in mg/dL.

Evaluation of Liver Function Parameters

The activity of AST in serum was determined by colorimetric assay from Sigma Diagnostics Laboratories. The method was based on the transamination reaction between L-aspartate and α-oxoglutarate to form oxaloacetate and L-glutamate. The 2,4-DNPH reacted with the oxaloacetate formed to form a coloured derivative of hydrazone. 100µL serum was diluted with 500µL of the included buffer and set at 37°C for 30 minutes for analysis. Then 500 µL of colour developing reagent was added and incubated at 25°C for 20 minutes. A certain amount of sodium hydroxide was added to stop the reaction and the absorbance was recorded at 546 nm using reagent blank. Enzymes were measured in units per litre (U/L).

The serum ALT activity was estimated by a commercial DiaSys diagnostic reagent kit. The assay was adapted to be based on the enzyme degradation of L-alanine and α-oxoglutarate into L-glutamic acid and pyruvate. The reaction was coupled to oxidation of NADH and the absorbance at 340 nm was monitored. The working reagent was made by mixing 4 parts of reagent R1 to 1 part of reagent R2. One hundred microliters of serum were added to one thousand microliters of the working reagent and incubated at 37°C, and absorbances were measured with the BTS-330 biochemical analyzer, and enzymatic activities were calculated according to the available calibration procedures. ALT was measured in units per litre (U/L).

Analysis of Renal Function Parameters

Serum urea, creatinine and uric acid was used to assess renal function. These parameters were incorporated in the biochemical estimation to observe difference between the various genotype group in cardiovascular patients in respect of renal function markers. Concentrations were reported in mg/dL and used for the statistical comparisons between genotypes.

Genomic DNA Extraction

DNA was isolated from whole blood in EDTA from which genomic DNA was extracted by phenol–chloroform–isoamyl alcohol extraction technique. Blood samples were processed by cellular lysis, then sodium dodecyl sulfate and proteinase K were added to degrade proteins and to release nucleic acids. The samples were kept at 65°C for about 3 hours prior to the organic extraction. After centrifugation, the aqueous layer (genomic DNA) was gently removed and placed in sterile microcentrifuge tubes. DNA was precipitated using 3 M sodium acetate and isopropanol, centrifuged and washed with 70% ethanol. DNA pellets obtained were air dried at room temperature and re-suspended in PCR grade water for further molecular analysis.

Assessment of Genomic DNA Quality

Genomic DNA was isolated and analyzed by agarose-gel electrophoresis. The agarose gel concentration was 1% in TAE buffer and the gel was stained with Ethidium Bromide. DNA samples were mixed with bromophenol blue loading dye and placed in the prepared gel wells. The electrophoresis was carried out at about 110 V for 30 minutes. After

electrophoretic separation, the integrity of the genomic DNA was evaluated with the bands of DNA under ultraviolet (UV) illumination before PCR amplification.

Molecular Genetic Analysis and Primer Design

The tetra-primer amplification refractory mutation system polymerase chain reaction (tetra-ARMS-PCR) method was used to perform molecular genetic analysis. Target nucleotide sequences and related genetic information were downloaded from publicly available molecular databases, such as the National Center for Biotechnology Information (NCBI), Ensembl and Online Mendelian Inheritance in Man (OMIM). Primer1 software was used to design primers. Each candidate polymorphic region was amplified with 4 primers (forward outer, reverse outer, forward inner, and reverse inner primers). Primer properties such as GC content, melting temperature, self-annealing and dimers were evaluated with OligoCalc and OligoAnalyzer software. In-silico PCR alignment with human genome was done to assess the specificity of the primers.

Polymerase Chain Reaction Amplification

The tetra-ARMS-PCR technique was used for the allele-specific amplification in a Bio-Rad T100 thermal cycler. The PCR reaction mixture consisted of the genomic DNA template, 10× PCR buffer, magnesium chloride, deoxynucleotide triphosphates, Taq DNA polymerase, and inner/outer primers. The annealing temperature of the primers were optimized by gradient PCR. The thermal cycling protocol was as follows: Hot start: 95 °C/5 min, followed by 30 cycles of denaturation at 95 °C/1 min, primer annealing at 72 °C/1 min and extension at 72 °C/1 min. The last extension step was carried out at 72°C for 7 minutes. The amplified products were then subjected to agarose gel electrophoresis to assess allelic-specific amplification pattern.

Agarose Gel Electrophoresis and Genotype Determination

Amplified PCR products were separated on 2% agarose gel in TAE buffer. About 20µL of PCR product was loaded with 2µL of bromophenol blue loading dye into each well of the gel. A molecular size reference was a 1000-bp DNA ladder from Thermo Fisher Scientific. The electrophoresis was carried out at 110 V for about 40 minutes. The amplified fragments were observed under the ultraviolet light and the sizes of the fragments were measured with reference to the molecular weight marker. To discriminate the genotypic components in the molecular analysis, allele-specific amplification patterns were used.

Statistical Analysis

IBM SPSS Statistics version 20 and MS Excel 2013 were used in the statistical analysis. Continuous biochemical measurements were summarized as means and standard deviations, whereas categorical variables, including genotype and allele distributions, were expressed as frequencies and percentages. The mean biochemical measurements for the cardiovascular patients and healthy controls were compared using student's t-test. Genotype and allele frequencies were tabulated for comparisons between the study groups. Chi-square statistics were reported for categorical genetic comparisons and odds ratios were calculated using MedCalc online odds ratio calculator. The p-values and test statistics were used to summarize the statistical results.

Ethical Considerations

The study was approved by the Institutional Ethical Review Committee of The University of Lahore, Lahore, Pakistan under approval No. IMBB/BBBC/24/45. Research was carried out in line with the ethical principles of the Declaration of Helsinki. All participants signed informed consent forms prior to enrolment.

RESULTS

***FOXO4* Gene Polymorphism Distribution in Cardiovascular Patients and Controls**

A total of 150 participants, comprising 100 patients with cardiovascular disease (CVD) and 50 healthy controls, were included in the study conducted in Faisalabad, Pakistan. Genotype distributions of two *FOXO4* polymorphisms, rs1404536455 (G/A) and rs1222386114 (T/C), were compared between the study groups. For rs1404536455, the GA genotype was predominant in both cardiovascular patients (70.0%) and controls (91.5%). The AA genotype occurred more frequently among CVD patients than controls (24.0% versus 8.5%), whereas the GG genotype was identified in 6.0% of patients and was absent among controls. An overall difference in genotype distribution was observed between the groups (exact $p = 0.010$).

For rs1222386114, the TT genotype was the most frequently observed genotype among cardiovascular patients (75.5%) and controls (84.0%), followed by the TC genotype (22.4% and 16.0%, respectively). The CC genotype was

identified in 2.0% of genotyped cardiovascular patients and was absent among controls. The overall genotype distribution did not differ significantly between the two groups (exact p = 0.409). The distributions of both polymorphisms are presented in Table 1 and Figure 1.

Table 1. Distribution of *FOXO4* gene polymorphisms among cardiovascular patients and healthy controls

Polymorphism / Genotype	Controls, n (%)	CVD patients, n (%)	Exact p-value
rs1404536455 (G/A)	**n = 47**	**n = 100**	**0.010**
GA	43 (91.5)	70 (70.0)	
AA	4 (8.5)	24 (24.0)	
GG	0 (0.0)	6 (6.0)	
rs1222386114 (T/C)	**n = 50**	**n = 98**	**0.409**
TC	8 (16.0)	22 (22.4)	
TT	42 (84.0)	74 (75.5)	
CC	0 (0.0)	2 (2.0)	

Values are expressed as frequencies and percentages calculated using the available genotype counts for each polymorphism. Exact p-values were calculated using the Fisher–Freeman–Halton test for the overall genotype distributions.

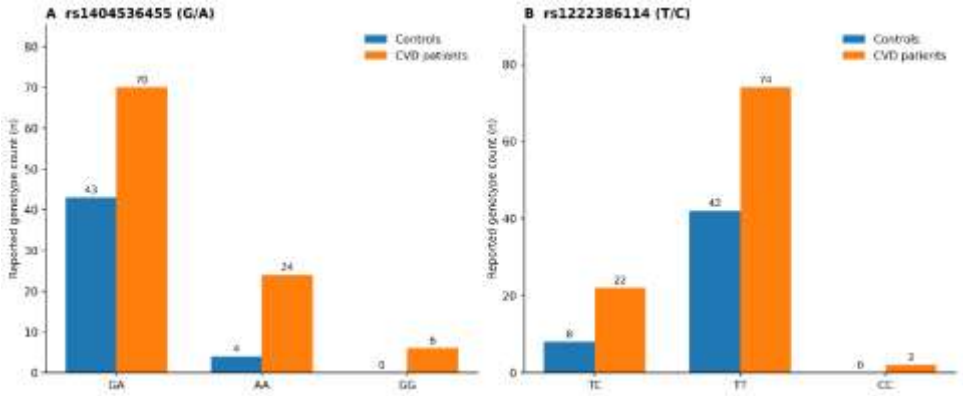

Figure 1. Distribution of *FOXO4* polymorphism genotypes among cardiovascular patients and healthy controls. (A) rs1404536455 (G/A); (B) rs1222386114 (T/C)

Association of rs1404536455 Polymorphism with Lipid Profile

Lipid profile parameters were compared among cardiovascular patients carrying the GA, AA, and GG genotypes of rs1404536455. Mean total cholesterol concentrations were 210.5 ± 40.3 mg/dL in GA carriers, 190.4 ± 35.2 mg/dL in AA carriers, and 220.7 ± 45.6 mg/dL in GG carriers (p = 0.069). Corresponding triglyceride concentrations were 210.9 ± 40.8, 190.6 ± 35.9, and 220.3 ± 45.5 mg/dL, respectively (p = 0.073). The GG genotype exhibited the highest mean concentrations of total cholesterol, triglycerides, HDL-C, and LDL-C. However, no statistically significant differences were observed across genotypes for total cholesterol, triglycerides, HDL-C (p = 0.339), or LDL-C (p = 0.472). The lipid profile findings are presented in Table 2 and Figure 2.

Association of rs1404536455 Polymorphism with Liver and Renal Function Parameters

Hepatic enzyme analysis demonstrated variations in serum ALT and AST activities across rs1404536455 genotypes. Mean ALT activity was 45 ± 8 U/L among GA carriers, 40 ± 7 U/L among AA carriers, and 47 ± 9 U/L among GG carriers, with a statistically significant overall difference (p = 0.019). Corresponding AST activities were 35 ± 6, 33 ± 5, and 36 ± 7 U/L, respectively, without a significant difference (p = 0.294). Renal biochemical assessment demonstrated the highest mean urea (32 ± 6 mg/dL), creatinine (1.1 ± 0.3 mg/dL), and uric acid (5.8 ± 0.8 mg/dL) concentrations among GG genotype carriers. A nominally significant overall difference was observed for serum creatinine (p = 0.048), whereas urea (p = 0.155) and uric acid (p = 0.081) did not differ significantly across genotype groups. The hepatic and renal biochemical findings are summarized in Table 2 and Figure 3.

Table 2. Association of rs1404536455 genotypes with biochemical parameters in cardiovascular patients

Parameter	GA (n = 70)	AA (n = 24)	GG (n = 6)	p-value
Lipid profile				
Total cholesterol (mg/dL)	210.5 ± 40.3	190.4 ± 35.2	220.7 ± 45.6	0.069
Triglycerides (mg/dL)	210.9 ± 40.8	190.6 ± 35.9	220.3 ± 45.5	0.073
HDL-C (mg/dL)	55.3 ± 10.1	52.7 ± 9.4	58.8 ± 11.2	0.339
LDL-C (mg/dL)	120.6 ± 20.5	115.8 ± 18.7	125.4 ± 22.3	0.472
Liver enzymes				
ALT (U/L)	45 ± 8	40 ± 7	47 ± 9	**0.019**
AST (U/L)	35 ± 6	33 ± 5	36 ± 7	0.294
Renal markers				
Urea (mg/dL)	30 ± 5	28 ± 6	32 ± 6	0.155
Creatinine (mg/dL)	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.3	**0.048**
Uric acid (mg/dL)	5.5 ± 0.7	5.2 ± 0.6	5.8 ± 0.8	0.081

Values are expressed as mean ± standard deviation. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

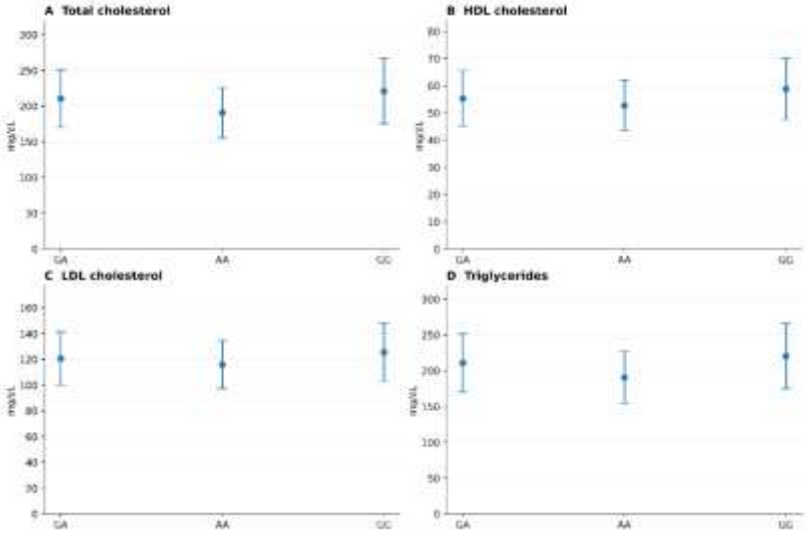

Figure 2. Lipid profile parameters across rs1404536455 genotypes: Panels A–D show mean ± SD concentrations of total cholesterol, HDL-C, LDL-C, and triglycerides among GA, AA, and GG genotypes

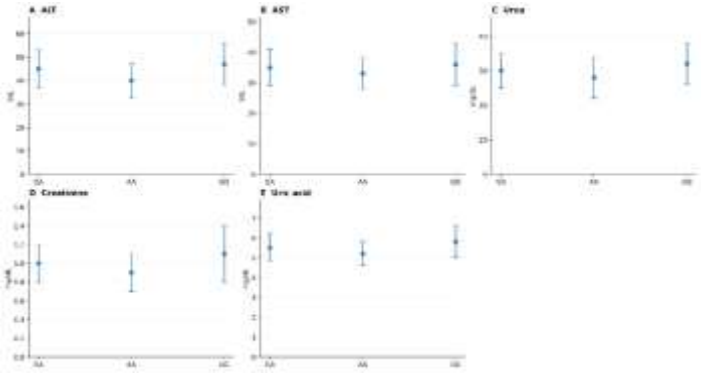

Figure 3. Hepatic and renal parameters across rs1404536455 genotypes: Panels A–E show mean ± SD values for ALT, AST, urea, creatinine, and uric acid

Association of rs1222386114 Polymorphism with Lipid Profile

The relationship between rs1222386114 and lipid profile parameters was examined across the TC, TT, and CC genotypes. Total cholesterol concentrations were highest among TT carriers (210.58 ± 30.21 mg/dL), followed by CC (205.34 ± 20.14 mg/dL) and TC carriers

(200.45 ± 25.17 mg/dL). Triglyceride concentrations demonstrated a similar distribution, with corresponding values of 160.64 ± 22.44, 155.89 ± 18.77, and 150.33 ± 20.18 mg/dL. LDL-C was also highest among TT carriers (110.25 ± 12.32 mg/dL), whereas HDL-C was highest among TC carriers (55.47 ± 8.23 mg/dL). No statistically significant differences were reported for total cholesterol (p = 0.55), triglycerides (p = 0.45), LDL-C (p = 0.55), or HDL-C (p = 0.65). The biochemical distributions are presented in Table 3 and Figure 4.

Association of rs1222386114 Polymorphism with Biochemical Parameters

Serum biochemical parameters were compared among cardiovascular patients carrying the TC, TT, and CC genotypes of rs1222386114. Mean total cholesterol, triglyceride, and LDL-C concentrations were highest among TT genotype carriers, whereas HDL-C concentrations were highest among TC carriers. Based on one-way ANOVA calculated from the group summary statistics, LDL-C differed significantly across genotypes (p = 0.007), while total cholesterol (p = 0.358), triglycerides (p = 0.157), and HDL-C (p = 0.630) showed no statistically significant differences.

Hepatic enzyme analysis demonstrated higher mean ALT activity among TT carriers (40.16 ± 6.21 U/L) compared with CC (38.41 ± 4.25 U/L) and TC carriers (35.22 ± 5.14 U/L). A significant overall difference was observed for ALT (p = 0.004), whereas AST activity did not differ significantly across genotypes (p = 0.465). Similarly, TT carriers exhibited the highest mean serum uric acid (6.47 ± 1.23 mg/dL), urea (32.13 ± 5.11 mg/dL), and creatinine (0.85 ± 0.12 mg/dL) concentrations. The overall difference was statistically significant for uric acid (p = 0.006), whereas urea (p = 0.384) and creatinine (p = 0.748) demonstrated no significant differences. The biochemical distributions are presented in Table 3 and Figure 5.

Table 3. Association of rs1222386114 genotypes with biochemical parameters in cardiovascular patients

Parameter	TC (n = 22)	TT (n = 74)	CC (n = 2)	p-value
Lipid profile				
Total cholesterol (mg/dL)	200.45 ± 25.17	210.58 ± 30.21	205.34 ± 20.14	0.358
Triglycerides (mg/dL)	150.33 ± 20.18	160.64 ± 22.44	155.89 ± 18.77	0.157
LDL-C (mg/dL)	100.12 ± 15.13	110.25 ± 12.32	105.73 ± 10.21	**0.007**
HDL-C (mg/dL)	55.47 ± 8.23	53.91 ± 6.14	54.35 ± 7.22	0.630
Liver enzymes				
ALT (U/L)	35.22 ± 5.14	40.16 ± 6.21	38.41 ± 4.25	**0.004**
AST (U/L)	30.31 ± 6.11	32.44 ± 7.33	31.79 ± 5.17	0.465
Renal markers				
Uric acid (mg/dL)	5.53 ± 1.01	6.47 ± 1.23	6.04 ± 0.84	**0.006**
Urea (mg/dL)	30.47 ± 4.25	32.13 ± 5.11	31.69 ± 3.18	0.384
Creatinine (mg/dL)	0.83 ± 0.11	0.85 ± 0.12	0.82 ± 0.08	0.748

Values are presented as mean ± standard deviation.

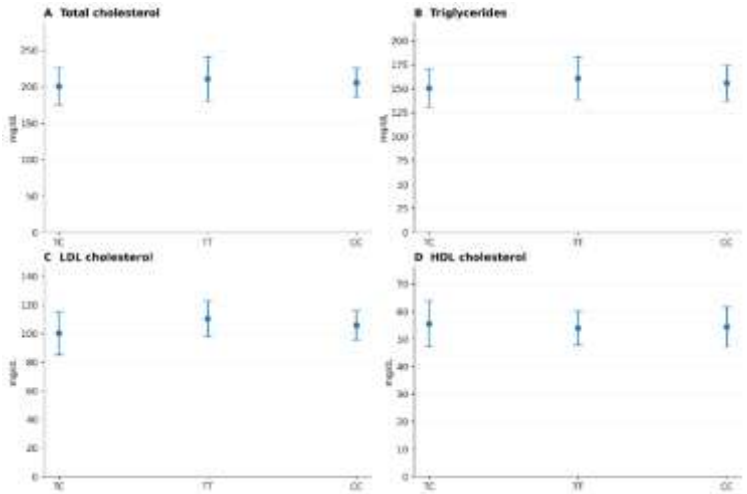

Figure 4. Lipid profile parameters across rs1222386114 genotypes: Panels A–D show mean ± SD values for total cholesterol, triglycerides, LDL-C, and HDL-C in TC, TT, and CC genotype groups

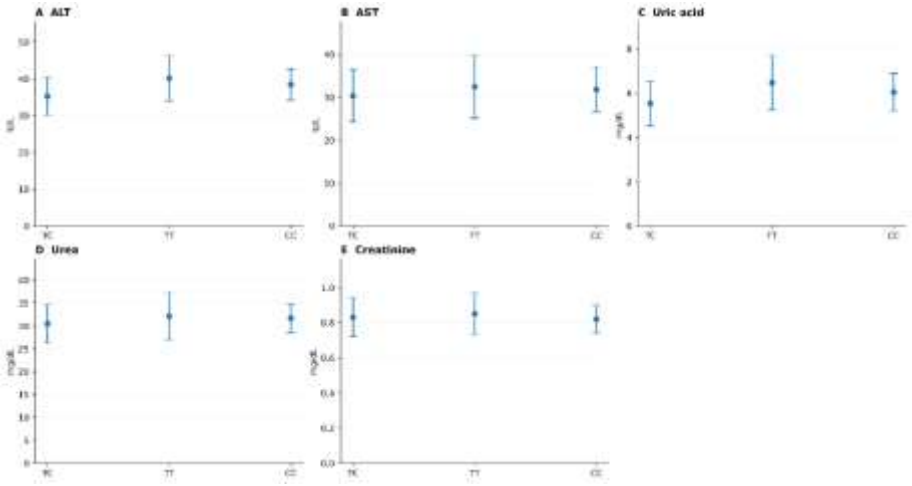

Figure 5. Hepatic and renal function parameters across rs1222386114 genotypes: Panels A–E show mean ± SD concentrations of ALT, AST, uric acid, urea, and creatinine

Across both polymorphisms, the biochemical measurements showed variations among genotype groups. The GG genotype was descriptively correlated with the highest mean values for the lipid, hepatic and renal parameters evaluated for rs1404536455. The mean levels of total cholesterol, triglycerides, LDL-C, liver enzymes and renal markers were generally higher among the carriers of TT genotype for rs1222386114. Study reported biochemical comparisons however yielded p-values > 0.05, and therefore no statistical evidence of difference in these measurements with regard to genotype (Tables 2 and 3; Figures 2 - 5).

DISCUSSION

The present study investigated the association of *FOXO4* gene polymorphisms rs1404536455 (G/A) and rs1222386114 (T/C) with cardiovascular disease (CVD) and selected biochemical parameters in individuals from Faisalabad, Pakistan. Genotype differences between cardiovascular patients and healthy controls were found[1,2]. AA genotype of rs1404536455 was more common in patients with CVD than controls (24.0% vs 8.5%) while the GA genotype was more common among controls (91.4% vs 70.0%). Likewise, the genotype TC of the rs1222386114 was found in 22.0% of cardiovascular patients and 16.0% of the controls. The results show differences in *FOXO4* genotype frequencies among the groups, and may indicate an association between genetic polymorphisms at these sites and cardiovascular risk. These differences suggest the need to study the genetic causes of cardiovascular disease in particular populations[3].

FOXO4 has been implicated in regulation of oxidative stress, inflammatory signaling, apoptosis, and cellular homeostasis, providing compelling evidence of its biological relevance in cardiovascular disease[4]. *FOXO4* has been implicated in various molecular pathways that regulate endothelial function, vascular smooth muscle cell activity and cardiomyocyte survival. Changes in these regulatory mechanisms can help result in vascular damage, myocardial remodeling, and cardiovascular dysfunction. Experimental data suggest that *FOXO4* is involved in cellular response to oxidative damage and inflammatory stimuli, which may be different depending on the cellular environment and disease mechanisms. The present study reveals differences in genotype distributions that are consistent with the general biological relevance of the *FOXO4*-mediated signaling in cardiovascular pathophysiology, but without showing a functional effect of the variants examined[5].

Lipid profile parameters were analysed and no significant relationship was found among the genotypes of the rs1404536455 and the serum levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) or low-density lipoprotein cholesterol (LDL-C)[6]. Persons with the GG genotype had relatively high mean values of some lipid parameters but the differences were not significant. Likewise, the mean total cholesterol, triglyceride and LDL-C concentrations in TT genotype carriers were comparatively high while HDL-C concentrations were marginally higher among TC. None of these differences was significant[7]. The metabolism of lipids is a complex regulation that is controlled by genetic, hormonal, nutritional and environmental factors. Thus,

changes in one transcription regulatory gene may not lead to changes in circulating lipids. The results showed that in this study population none of the *FOXO4* polymorphisms were significantly associated with conventional lipid profile parameters[8].

Similar results were found with liver enzymes and kidney function tests. However, there were only slight differences among the *FOXO4* genotypes studied in terms of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, and these differences were not statistically significant. Likewise, there was no significant association between each polymorphism and serum urea, creatinine and uric acid levels[9]. Various physiological and pathological conditions such as metabolic control, inflammation, drug exposure, and cardiovascular hemodynamics affect the activity of hepatic enzymes and renal biochemical parameters. Because no significant difference between the genotypes was found, these traditional biochemical parameters showed no measurable association with the *FOXO4* variants studied in this population[10].

Of particular interest was the difference in the distributions of the genotypes among cardiovascular patients and controls, in the absence of statistically significant associations with the biochemical parameters analyzed[11]. This suggests that genes that are associated with cardiovascular susceptibility do not always change levels of lipids, liver or kidney parameters that are currently measured. *FOXO4* is implicated in molecular pathways involved in oxidative stress regulation, endothelial function, inflammatory responses, and cellular apoptosis, all of which are key players in cardiovascular pathophysiology. These mechanisms could be used to give the observed genetic patterns a biological context but the case–control design does not provide causality. The results highlight the difference between the genetic variation related to disease status and to the traditional biochemical traits[12].

A major strength of the current study was that both genetic polymorphisms of *FOXO4* and various biochemical parameters were studied in cardiovascular patients and healthy controls from Faisalabad, Pakistan[13]. Combination of molecular genetic investigations and biochemical profiling was used to provide a wider assessment of potential relationships between *FOXO4* variation and cardiovascular disease. However, interpretation of the effects of the subgroup comparisons must take into account the relatively small numbers of people with uncommon homozygous genotypes. Association may be affected by differences in age, sex, cardiovascular risk factors, medication exposure, and underlying metabolic conditions as well. The latter is especially important in the context of genetic association studies in a heterogeneous cardiovascular population. In addition, sex-specific genetic characteristics need to be taken into account when assessing genotype distributions because *FOXO4* is X-linked[14,15].

The results provide population-specific data on cardiovascular disease association and genetic variability of *FOXO4* gene[16]. The observed genotype differences, along with the lack of significant variants with commonly measured biochemical parameters, indicate that the association between variation in *FOXO4* and susceptibility to cardiovascular disease might be linked to molecular pathways not typically examined by biochemical measures[16,17]. Sex-stratified genetic analysis and molecular analysis of oxidative stress and inflammatory pathways in larger population-based investigations with detailed cardiovascular phenotyping might help to clarify the biological relevance of the *FOXO4* polymorphisms in cardiovascular disease[18-20].

CONCLUSION

The present study identified differences in the distribution of *FOXO4* polymorphisms rs1404536455 and rs1222386114 between cardiovascular patients and healthy controls in Faisalabad, Pakistan. Polymorphism of both did not show any statistically significant association with lipid profile parameters, hepatic and renal function markers. These results offer insights on genetic variability of *FOXO4* in the population and its potential association with cardiovascular susceptibility and the role of molecular genetic factors in cardiovascular research.

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