Association of Angiotensin Converting Enzyme (ACE) Gene Polymorphisms and Risk of Diabetic 2 Among Patients Visiting Bahirdar Felegehiwot Referral Hospital North West, Ethiopia

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Received date: February 20, 2018; Accepted date: February 27, 2018; Published date: March 07, 2018

Abstract

Background

Type 2 diabetes (T2DM) affect large population. There are many genetic and non genetic factors associated for the occurrence of T2DM. Genes of the renin angiotensin system angiotensin converting enzyme insertion/deletion gene polymorphism has been associated with the risk of type 2 diabetes. The aim of the present study was to investigate the association of angiotensin converting enzyme gene polymorphism, risk of type 2 diabetes.

Methods

A total of 222 subjects (111 T2DM and 111 healthy controls) were collected from Bahir Dar Felegehiwot Referral Hospital and Bahir Dar town, respectively by using non-probability purposive sampling techniques. Minidray fully automated analyzer was used for biochemical tests. The ACE I/D genotypes were identified by Polymerase chain reaction (PCR) using appropriate primers and PCR reaction conditions.

Results

The present study revealed that the frequency of DD genotype and D allele were higher in type 2 diabetes mellitus compared to healthy controls (DD, 64.2% vs.35.6% P<0.001) and (D, 79.3% vs.59.9% P<0.001). DD genotype showed threefold increase risk towards T2DM as compared to II Genotype (OR: 2.984, CI: 1.332-6.689, P<0.02).

The D allele carriers had five times high risk of getting diabetic as compared to I allele. (OR, D 2.178; CI: 1.168-3.232 P<0.001 vs.I OR, 0.459; CI: 0.309-0.681 P<0.001).

Conclusion

This study indicated that the frequency of ACE DD genotype and D allele were markedly higher in patients with type 2 diabetes.

Keywords: Diabetes; T2DM; ACE gene polymorphism; Genetic polymorphism

Background

Diabetes is a rising global health burden, which is a multifactorial, heterogeneous group of metabolic disorder with the common feature characterized by a deficiency or failure in maintaining normal glucose homeostasis; this is due to defective insulin secretion, resistance insulin action or due to a combination of both [1].

Diabetes mellitus is a serious condition with potentially stressful complications that affects all age groups worldwide [2]. The American Diabetes Association (ADA) divides diabetes mellitus into four categories [3]. Type 2 diabetes mellitus (T2DM) is the second category and most diabetes is sorted in this category, a heterogeneous group of disorders caused by some combination of insulin resistance and impairment of insulin secretion [3]. Insulin resistance is a condition in which the body's muscle, fat and liver cells does not use insulin effectively leads to high concentration of glucose in the circulation and occurred when the body can no longer produce enough insulin to compensate for the impaired ability to use insulin [4-6].

According to reports by International diabetic federation (IDF), in 2013 approximately 382 million people had diabetes globally and the IDF has estimated this number will rise to 592 million by 2035 [2]. Among those T2DM, accounts about 350 million people worldwide and estimated 10 percent of the world's adult population (nearly 600 million people) will suffer from the disease by 2035 [7]. However, the largest increases of population with diabetes occur in sub-Saharan Africa, with a projected growth of 19.8 million in 2013 to 41.5 million by 2035 [2]. Among this, over 90% are T2DM in Sub-Saharan Africa [8]. This is due to a rapid uncontrolled urbanization, lifestyle changes...
towards western diets, reduced quality of food, late diagnosis, inadequate screening and diagnostic resources, poor control of blood sugar level, inadequate treatment at an early stage and smoking in sub-Saharan Africa [9-11]. Since 1985 Ethiopia has been a member of IDF and the IDF estimated the number of diabetic among adults (20-79 years) in 2010 was 2.5% and the number is expected to rise in to 3.5% by the year 2030 [12]. Currently diabetes is a serious challenge in Ethiopia [13-15] and undiagnosed cases (late diagnosis) in Ethiopia is highest proportion (82.6%) among the rural population and 63% among the urban population and family history of diabetes is highly associated with diabetes mellitus [16].

It is typically a multifactorial disease, there are many factors associated in the occurrence of T2DM and the progress of its complication. Risk factors such as obesity (Body mass index (BMI)), over eating, stress as well as aging, dyslipidemia, hypertension, smoking, physical inactivity, dietary patterns, family history, and specific genes are the most frequently known risk factors for T2DM [17-19]. However, the genetic factors (genetic origins) of T2DM and its complication are not obviously understood because the genetics of T2DM is polygenic and multifactorial origins as a result of the interaction between the environment and multiple genes [11,20].

At present many candidate genes have been associated with T2DM [21]. However, genes that are encoding for the renin-angiotensin system (RAS) are the main genetic risk factors for T2DM and the progress of its complications [22,23]. Among genes involved in the RAS, Angiotensin converting enzyme (ACE) gene encoding is a key enzyme. This gene is located at chromosome 17q23 consisting of 26 exons, 25 introns and it spans 21 kb [24]. The polymorphism of ACE gene due to the insertion (I) or deletion (D) of a 287 bp Alu repeat sequence near the 3’ end of intron 16 leads to three genotypes DD, II and ID [25,26]. The main function of this gene is the conversion of Angiotensin I to vasoactive, natriuretic octapeptide angiotensin II in liver [24] and inactivates a vasodilator peptide bradykinin [27]. Alleles of ACE gene polymorphism are present in both healthy individuals and T2DM patients with different frequencies and DD genotype individuals are at risk of T2DM [28]. This implies that, imbalances in RAS functions are related to insulin resistance and susceptible to T2DM. The present state of knowledge about ACE gene polymorphism in T2DM and its complication have a doubt because there were some studies in the literature that described a null association between the etiologies of T2DM with ACE gene polymorphism [23,29]. However, most of the reported literature has indicated that ACE gene polymorphism is associated with T2DM [30-32]. However, there were no reported data of ACE gene polymorphism on the risk of T2DM among the Ethiopian population. Therefore, the aim of this study was to investigate the association of risk factors, ACE gene polymorphism in patients with T2DM among the Ethiopian population, Bahir Dar Felegehiwot Referral Hospital.

Material and Methods

Study area

This study was conducted in Bahir Dar Felegehiwot Referral Hospital. Bahir Dar town is located in the Amhara region, Northwest of Ethiopia served by Felegehiwot Referral Hospital, a tertiary referral hospital serving over 7 million people from the surrounding area including Debre tabor hospital, Woreta hospital, Dangla hospital, Durbette hospital, Merayi hospital, Adiet hospital, Enjibara hospital, Gemjabet hospital, Burie hospital, Chagni hospital, Mota hospital. For this study, samples were collected from the diabetic clinic of Bahir Dar Felegehiwot Referral Hospital. The biochemical analysis of the study was done in Gondar University Referral Hospital, clinical diagnostic laboratory. However, the molecular part of the experimental analysis was conducted in Gondar University Atse Tewodros Campus, College of Natural and Computational Sciences, Department of Biotechnology.

Study design and period

A case control comparative study was carried out from January–October, 2016 in Bahir Dar Felegehiwot Referral Hospital.

Population

During the study period, all patients who were diagnosed by physicians according to the WHO criteria for diabetes mellitus and healthy control subjects were the source population. Whereas T2DM patients with or without complication who were diagnosed by physicians were as the study population.

Inclusion and exclusion criteria

Patients diagnosed by physicians having type 2 diabetes and all age groups were recruited in the study. Patients were diagnosed with T1DM, patients suffering from acute and chronic infection, malignancies, congestive heart failure, HIV, urinary tract infection and acute febrile illness were excluded from this study. Healthy controls having hypertension at the time of data collection and healthy controls having hyperglycemia during biochemical analysis were excluded in the study and replaced by other healthy individuals.

Variables

The dependent variables in these studies were age, sex, duration of diabetics and status of diabetes. Whereas the independent variables include serum creatinine, glucose level, cholesterol, triglycerol, blood urea, ACE gene ID/ID polymorphism, and obesity.

Sample size and sampling technique

A total of 222 subjects (111 T2DM and 111 healthy controls) were selected by non probability purposive sampling technique Among T2DM, 55 were men and 56 were women and healthy control groups consisted, 55 men and 56 women.

Data collection, Clinical Measurements and Laboratory Method

Questionnaire

The socio-demographic characteristics and clinical parameters of both patients and healthy control subjects such as smoking, gender, age, duration of diabetes, and family history were taken through semi-structured questioner.

Anthropometric measurements

Body weight was measured using a portable digital scale while height was measured using a portable stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters and participants were categorized as
underweight<18.5, healthy (BMI 18.5-25 kg/m2), overweight (BMI 25.0-29.9 kg/m2) or obese (BMI ≥ 30 kg/m2) [33]. Blood pressure was recorded in the sitting position after 5 min of rest by using a digital device and systolic and diastolic blood pressures were calculated from mean value after three readings. Patients were considered as hypertension, if mean systolic blood pressure (SBP) ≥ 140 mmHg and mean diastolic blood pressure (DBP) ≥ 90 mmHg or if they used antihypertensive medication.

Sample collection

Five milliliters of blood sample were collected from the vein of all subjects of an overnight fasting patients and healthy controls by laboratory personnel. From 5ml blood sample, 3 ml was kept in plain tube without anticoagulants for biochemical tests. The remaining 2 ml of the blood was kept in Ethylene Diamine Tetra Acetic Acid (EDTA) tube for isolation of DNA and stored at -21°C.

Laboratory methods

Biochemical analysis: A non–anticoagulated blood (3 ml in plain tube) sample was centrifuged at 8000 rpm for 10 mins, the sera were aspirated and transferred in to sterilized eppendorf tube and stored at -20°C until processing. Each test was subjected for an enzymatic analysis of glucose, total cholesterol, triglycerides, urea, and creatinine by Mindary fully automated analyzer in the clinical chemistry diagnostic laboratory of college of medicine and health sciences. Fasting blood glucose (FBG) level was used to assess the glycemic control. For a non-diabetic individuals (healthy control group), when FBG was >110 mg/dl, the glycemic control was considered as unsatisfactory; when FBG ≤ 110 mg/dl the glycemic control was considered as satisfactory [34]. However, in the case of diabetic patients when FBG>130 mg/dl, the glycemic control was considered poor glycemic control. If total cholesterol>200 mg/dl and triglycerides>150 mg/dl, were taken as abnormal [36]. If serum creatinine>1 mg/dl, it was taken as abnormal result [7]. Estimated glomerular filtration rate (eGFR) was calculated online by using the Chronic Kidney Disease Epidemiology Program equation (CKD-EPI) and individuals having eGFR<60 ml/min/1.73m² were considered as having a chronic kidney disease [37]. Moreover, based on eGFR, individuals were classified as risk categories (low, moderate, high or very high) by the Kidney Disease Improving Global Outcomes (KDIGO) 2012 prognostic guidelines [38].

Genomic DNA isolation: For DNA extraction, 300µl of EDTA anticoagulated bloods of both patients and healthy controls was transferred in to sterilized 1.5ml eppendorf tube. A non-enzymatic salting out method was used for DNA extraction to get high yield genomic DNA with a small amount of blood sample [39]. Red blood cells (RBCs) were lysed and removed by using RBC lysis buffer solution. Similarly, white blood cells were lysised using a nuclear lysis buffer solution. The proteins were precipitated with a high concentrated salt (6M NaCl) and DNA was precipitated by chilled absolute ethanol, followed by washing of genomic DNA with 70% ice-cold ethanol and finally the DNA was dissolved with Tris-EDTA buffer (TE) and this sample was stored at -21°C till used.

The quality of isolated genomic DNA was confirmed by using 1.5% agarose gel electrophoresis. Finally via following the above mentioned procedures a pure genomic DNA was obtained as shown in the Figure 1. Then the patients and respective control samples were genotyped for ACE Insertion/Deletion (I/D) polymorphism using sets of primers and appropriate PCR conditions.

Polymerase chain reaction (PCR): The Insertion/Deletion (I/D) alleles of ACE gene polymorphisms were identified by TC 412 PCR amplification of using specific primers. A final volume (25 µl) of PCR reaction mixture were prepared using 6µmol of forward and reverse primers (forward primer 5'-CTGAGACCACCTCCATCTTTCTTCT-3' and reverse primer 5'GATGTGGCCATCATTCGTCTGAT-3') [40,41], 5 mM of MgCl2, 0.5 mM of each dNTP, 0.75 U of hot start Taq polymerase, 3 µl of template DNA and water were used.

PCR amplification was set with an initial denaturation at 95°C for two minutes. Hot start Taq polymerase was kept for 15 minutes at 95°C to activate the enzyme. Then, the DNA was amplified for 30 cycles. The cycle steps were denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 45 seconds followed by a final extension at 72°C for nine minutes. Finally the PCR product was held at 4°C until it was analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis: PCR amplified products of ACE I/D genotypes were electrophoretically separated for 50min at 50 V on a 2% agarose gel. To stain and visualize DNA upon UV transillumination in gel, 3 µl of 2% Ethidium Bromide was also added. The PCR amplified products (12 µl) mixed with 3 µl loading dye then loaded into wells of agarose gel. Electrophoresis was carried out in 1X trice acetate EDTA (TAE) buffer and the gel was visualized by UV transilluminator.

After electrophoresis, band sizes of 190bp (Deletion) and 490bp (Insertion) polymorphisms fragments were obtained and image was captured with the help of a smart phone camera and digital camera. Therefore, there were three genotypes after electrophoresis: A 490bp band (genotype II), a 190bp band (genotype DD) and both 490bp and 190bp band (ID genotype). The genotypes were calculated to determine the association of ACE (I/D) gene polymorphisms in patients with T2DM, DR, DN, diabetic with hypertension and with other clinical and socio-demographic characteristics.

Statistical analysis: Data were analyzed by using SPSS version 20. Quantitative data were presented as mean and standard deviation (x ± s). To compare continuous variables between T2DM group and healthy control groups, T2DM and T2DM complication, t-test for independent samples was used. Distribution of the genotype and allele frequencies
in T2DM and healthy control groups, T2DM and T2DM with complications were compared using chi-square test. The risk associations of DD genotype and D allele with T2DM were assessed by the odd ratio at 95% confidence interval (CI). Relationships between the ACE genotypes and clinical variables were compared with one-way analysis of variance (ANOVA).

Ethical consideration: The study protocol was approved by the college of natural and computational sciences ethical committee of the University of Gondar on its meeting held on 11/08/08 ethical clearance. To take care and keep confidentiality of participants result, written informed consent was obtained from all study participants before collecting of blood sample.

Results

Demographic, clinical and biochemical characteristics between t2dm and healthy controls

The demographic parameter of study subjects is given in Table 1. There was no statistically significant variation between cases and controls in respect to age and sex. Among cases, 55 were men and 56 were women, (P = 0.789), their mean age was 54.6 ± 12.2 years. The control group consisted, 55 men and 56 women their mean age was 54.0 ± 14.4 years, (P<0.811). The clinical characteristics systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, total cholesterol, triglycerol and urea have shown a significant difference (P<0.001), BMI (P<0.05) and creatinine (P<0.02) higher in T2DM than control group. However, eGFR was not significantly differed between the two groups (P=0.052).

| Variables   | T2DM     | Control    | P value |
|-------------|----------|------------|---------|
| Subject (n) | 111      | 111        | -       |
| Gender (M/F)| 55/56    | 55/56      | 0.789   |
| Age (yr)    | 54.7 ± 12.3 | 54.3 ± 14.4 | 0.811   |
| BMI (kg/m2) | 24.9 ± 4.4   | 23.6 ± 5.4   | 0.049   |
| SBP (mmHg)  | 133.7 ± 19.5 | 121.2 ± 6.9   | 0       |
| DBP (mmHg)  | 82.6 ± 13.6   | 76.4 ± 5.5   | 0       |
| Glucose (mg/dl) | 206.3 ± 72.3 | 78.2 ± 15.6 | 0       |
| TC (mg/dl)  | 194.81 ± 47.6 | 130.4 ± 48.9 | 0       |
| TG (mg/dl)  | 177.5 ± 135.3 | 99.6 ± 56.7  | 0       |
| Creatinine (mg/dl) | 0.7 ± 0.2     | 0.6 ± 0.1  | 0.01    |
| Urea (mg/dl) | 22.2 ± 8.3 | 18.8 ± 4.6 | 0       |
| eGFR (ml/min/1.73m2) | 118.4 ± 27.1 | 124.7 ± 20.2 | 0.052   |

Table 1: Demographic, Clinical and biochemical characteristics of T2DM patients and healthy controls (Data expressed as means ± SD; P<0.05 were considered as significant and P>0.05 (not significant) Note: BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; TC: Total Cholesterol; TG: Triglycerol; eGFR: Estimated Glomerular Filtration Rate).

ACE genotype distribution in T2DM patients and healthy controls

The ACE genotypes frequency distribution in patients with type 2 diabetes and healthy control is given in table 2. The ACE DD genotype and D allele were more frequent in diabetic patients (64.2% and 79.3%) as compared to controls (35.6% and 59.9%). The frequency of homozygous DD genotype in type 2 diabetic patients was twofold higher than healthy control group (OR, 2.984; CI 1.332-6.689). D allele was five times than I allele in diabetic patients (OR, D 2.178; CI 1.168-3.232 P<0.001) compared to healthy controls (I OR, 0.459; CI: 0.309-0.681 P<0.001).

However, ACE genotype I/D and I/I were less frequent in T2DM patients (31.4% and 37.5%) in comparison to healthy controls (69.0% and 62.5%).

| Variables | T2DM | Control | OR | OR 95% CI | P    |
|-----------|------|---------|----|-----------|------|
| DD        | 77 (64.2%) | 42 (35.6%) | 2.984 | 1.332 - 6.689 | 0.008|
| ID        | 22 (31.4%) | 49 (69.0%) | 0.7641 | 0.318 - 0.834 | 0.547|
| I         | 12 (37.5%) | 20 (62.5%) | 0       | 21.290a     |

Table 2: Distribution of ACE genotypes and allele frequencies between T2DM and healthy control participants [P<0.001 (statistically significant)].
Association of ACE I/D genotype with clinical and biochemical characteristics of T2DM and healthy controls

The ACE DD genotype was highly associated with the clinical variables than I/D and II genotypes. SBP (131.5 ± 18.9 vs. 123.2 ± 8.3 and 121.3 ± 10.7), glucose (168.2 ± 86.8 vs. 110.5 ± 61.9 and 114.7 ± 75.6), total cholesterol (179.2 ± 56.8 vs. 141.2 ± 57.1 and 145.0 ± 42.7), triglycerol (167.5 ± 136.3 vs. 102.4 ± 51.8 and 109.3 ± 53.9) and urea (22.7 ± 7.6 vs. 17.9 ± 5.1 and 17.8 ± 4.3) (P<0.02) respectively (Table 3 and Figure 2).

Table 3: One-way ANOVA analysis of clinical and biochemical characteristics according to ACE genotype in T2DM and healthy controls (Data expressed as means ± SD, P<0.05 were considered as significant and P>0.05 (not significant) Note: SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; Glu: Glucose; TC: Total Cholesterol; TG: Triglycerol; CRT: Creatinine; eGFR: Estimated Glomerular Filtration Rate; BMI: Body Mass Index.)

| Genotype result | Age     | SBP     | DBP     | Glu     | TC     | TG     | CRT     | Urea    | eGFR    | BMI     |
|-----------------|---------|---------|---------|---------|--------|--------|---------|---------|---------|---------|
| DD              | 54.9±12.8| 131.5±18.9| 81.9±12.4| 168.2±86.8| 179.2±56.8| 167.5±136.3| 0.697±.24| 22.7±7.6| 120.2±27.8| 24.9±4.4|
| ID              | 54.6±14.2| 123.2±8.3  | 77.4±6.5  | 110.5±61.9| 141.2±57.1| 102.4±51.8| 0.644±.2  | 17.9±5.1 | 122.9±18.4| 24.2±5.8 |
| II              | 52.3±13.9| 121.3±10.7 | 75.2±9.6  | 114.7±75.6| 145.0±42.7| 109.3±53.9| 0.699±.18 | 17.8±4.3 | 123.6±19.5| 22.0±4.3 |
| p-value         | 0.001   | 0       | 0.001   | 0       | 0     | 0.025  | 0       | 0.063   | 0.014   |

Discussin

Type 2 diabetes mellitus is a metabolic disorder of chronic disease that leads to the development of micro and macrovascular complications such as DR, DN, diabetic neuropathy and cardiovascular disease [18,19]. Genetic or non-genetic factors are able to predispose to diabetes and facilitate the progression of diabetic complications, as reported by different authors [11,14,15]. The non-genetic risk factors such as BMI, age (oldest age), life style, smoking, dyslipidemia, and environmental factors are the most common risk factors for T2DM [42]. There are many candidate genetic variants associated to the occurrence of T2DM [43]. However, genes of the RAAS such as renin, angiotensinogen (AGT), angiotensin-1 converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), aldosterone synthase (CYP11B2), angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors all of these genes or genetic loci responsible for excess Ang II production and Ang II has the most common physiological effect on T2DM [44,45].

The ACE I/D polymorphism has a potential link to diabetes and able to assist the progress of diabetic complications [46,47]. However, the ACE gene polymorphism and risk of T2DM has conflicting reports. In this study, ACE gene polymorphism and associated risk factors in patients with T2DM with a healthy control group were studied. It was found that the frequency of DD genotype as well as D allele has shown statistically significant increase in diabetic patients in comparison to healthy control groups (64.2% and 79.3% vs. 35.6 and 59.9% P<0.001) respectively as shown in Table 3 and Figure 3. DD genotype carriers have shown more than twofold increased risk of developing T2DM as compared to II genotype carriers (OR: 2.984, CI: 1.332-6.689, P<0.02). D allele was five times higher than I allele associated to diabetic patients than healthy controls (OR, D 2.178; CI: 1.168-3.232 P<0.001 vs. I OR, 0.459; CI: 0.309-0.681 P<0.001). This finding is in agreement with some other studies, which indicated DD genotype is associated with high increased risk of T2DM and D allele has high relative risk towards developing type 2 diabetes mellitus [48-50] and while others reported that there was no association between ACE DD genotype and T2DM [31,51-53].

Whenever the genotype distribution and these clinical parameters were compared, there were statistically significant association between the DD genotype holders and the amount of SBP, TC, TG and urea (P<0.01), DBP (P<0.01), and BMI (P<0.02) among T2DM patients compared to healthy control groups having the same genotype holders. This is disagree with some other reports by Choudhry et al. [51] and Degirmenci et al. [53] indicated that clinical characteristic of T2DM patients such as age, BMI, SBP, DBP, hemoglobin A1C (Hba1C), TC, TG, HDL cholesterol levels, duration of diabetes, FBG and random blood sugar (RBS) level according to ACE I/D genotypes are no significance difference [51,54-57]. Similarly the study in USA showed that BMI, waist, fat-free mass, and physical activity did not differ by ACE genotype [58]. On the other hand, in this study some of the clinical variables such as age, FBG, creatinine and eGFR did not showed statistically significant association towards ACE genotype holders between study case groups and healthy controls. This is harmony with the study in USA, age and fasting glucose are not associated with ACE gene polymorphism, but 2h glucose levels are higher in DD than ID and II subjects of T2DM [58]. Cigarette smoking is a risk factor for T2DM, diabetic complications and DD genotype are
greater risk [59]. However, in this study there were no any cases that are smoking cigarettes.

Conclusion

In conclusion, the present study indicated that ACE gene of DD genotype and D allele have been associated with high risk of diabetes patients (OR: DD 2.984, CI: 1.332-6.689, P<0.02 and D: 2.178; CI: 1.168-3.232 P<0.001 vs. I, 0.459; CI: 0.309–0.681 P<0.00). Moreover, this genotype and allele was associated with some of the clinical Parameters such as age, SBP, DBP, glucose, total cholesterol and urea. DD genotype have had high relative risk of getting diabetic as compared to ID and II genotype holders. The genotype (ID and II) and I allele in the healthy control group were high as compared to the cases. Some of the associated risk factors, DD genotype and both risk factors and DD genotype were associated to T2DM, which indicates the need for doing a similar study with a larger sample size to unravel the independent associated risk factors and ACE gene among T2DM and associated clinical variables. This study has some limitations: The assay for LDL and HDL could not done, those tests could have given a better estimate of lipoproteins. The tests were used only one primer for the detection ACE genotype ID/D, it is better to use a second primer after electrophoresis to increase the specificity of DD genotyping.

Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors’ contributions

TT, NB and WS participated in selecting the study area and preparing the proposal. TT and WS were carried out the experiment and NB supervised the overall activities and reviewed the documents. All authors have read and approved the final manuscript.

Acknowledgements

We would like to thank University of Gondar for funding this research and Institute of Biotechnology for providing us all the facilities during laboratory activities. We would like to acknowledge Bahirdar Felegehiwot Referral Hospital laboratory workers who help us in the data collection process. We would like to thank for clinical chemistry laboratory workers, college of medicine and health science, University of Gondar for analysis of biochemical tests. Our thanks also go to deep appreciation to Dr. Deepack Kumar Verma for import of primers from India. Moreover, we would like to express our sincere gratitude to Mr. Workie Anley, in Department of Plant Science, and Mr. Gashaw, in statistics department, University of Gondar for their support and cooperation in data analysis techniques. Finally yet mentioned here, yet lent their hand to us by any means during our studies.

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