Clinical Implications of MiR128, Angiotensin I Converting Enzyme and Vascular Endothelial Growth Factor Gene Abnormalities and Their Association with T2D

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Abstract: Type 2 DM (T2D) results from the interaction of the genetic and environmental risk factors. Vascular endothelial growth factor (VEGF), angiotensin I-converting enzyme (ACE), and MicroRNAs (MiRNAs) are involved in important physiological processes. Gene variations in VEGF, ACE and MiRNA genes are associated with diseases. In this study we investigated the associations of the VEGF-2578 C/A (rs699947), VEGF-2549 insertion/deletion (I/D), and ACE I/D rs4646994 and Mir128a (rs11888095) gene variations with T2D using the amplification refractory mutation system PCR (ARMS-PCR) and mutation specific PCR (MSP). We screened 122 T2D cases and 126 healthy controls (HCs) for the rs699947, and 133 T2D cases and 133 HCs for the Mir128a (rs11888095). The results showed that the CA genotype of the VEGF I/D polymorphisms were associated with T2D with OR =2.01, p-value = 0.011, and OR = 2.42, p-value = 0.010, respectively. The result indicated the D allele of the ACE ID was protective against T2D with OR = 0.10, p-value = 0.0001, whereas the TC genotype and the T allele of the Mir128a (rs11888095) were associated with increased risk to T2D with OR = 3.16, p-value = 0.0001, and OR = 1.68, p-value = 0.01, respectively. We conclude that the VEGF rs699947 and D allele of the VEGF I/D polymorphisms were associated with T2D with OR =2.01, p-value = 0.011, and OR = 2.42, p-value = 0.010, respectively. The result indicated the D allele of the ACE ID polymorphism may be protective against T2D. These results help in identification and stratification for the individuals that at risk for T2D. However, future well-designed studies in different populations and with larger sample sizes are required. Moreover, studies to examine the effects of these polymorphisms on VEGF and ACE proteins are recommended.

Keywords: type 2 diabetes mellitus (T2D); genome wide association studies (GWAS); vascular endothelial growth factor (VEGF); VEGF insertion and deletion (I/D); VEGF rs699947; ACE I/D rs4646994; Mir128a (rs11888095)
1. Introduction

Chronic diseases have often serious impact on the patients, their families and societies. Choosing an appropriate clinical investigation methodology is always essential to decide on proper treatment for patients [1,2]. Diabetes mellitus was one of the leading global causes of death in 2017 [3]. According to the WHO, Kingdom Saudi Arabia (KSA) ranks second in terms of DM prevalence and it has been estimated that more than 20% of Saudi population are diabetic [4]. This rate may be one of the highest in the world [4]. DM has very serious complications and consequences, such as diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, cardiovascular diseases and amputation. DM is a metabolic disorder characterized by hyperglycemia. The insufficient insulin secretion by the pancreatic beta cells leads to the development of type 1 DM (T1DM) [5]. Type 2 diabetes (T2DM) results from the combination of insulin resistance initiated in major tissues, excessive hepatic production of glucose and pancreatic beta cells’ dysfunction [6]. T2DM represents more than 90% of all cases of DM [7]. T2D is induced by complex interactions of genetic and environmental risk factors [8]. Genome wide association studies (GWAS) have uncovered the association of certain loci with metabolic diseases including T2D [9–15]. Environmental risk factors of T2D include obesity, physical inactivity and unhealthy diet [7]. The vascular endothelial growth factor (VEGF) is a growth factor expressed in endothelial cells and is involved in angiogenesis [16]. The VEGF promoter gene variations (-2578C/A and −1154G/A) have been associated with metabolic syndrome in the Korean population [17]. Moreover, the VEGF rs10738760 (A/G) SNP was associated with metabolic syndrome in Iranian and Lebanese populations [18,19]. Angiotensin I-converting enzyme (ACE) is a nonspecific peptidase with multiple peptide substrates [20]. The ACE converts the angiotensin-I into angiotensin-II [20]. This conversion leads to the activation of the angiotensin-I peptide hormone into the vasoconstrictor angiotensin-II [21]. ACE is involved in important physiologic processes such as blood pressure, development and function of the kidney, reproduction, and the formation of blood cellular components [20]. The long-term inhibition of the ACE has been suggested as one of the preventive measures against T2D [22]. Furthermore, the ACE gene variations were associated with the progression of carotid artery disease in Slovenian T2D patients [23]. MicroRNAs are short noncoding RNA molecules that are involved in the regulation of gene expression [24,25]. It has been reported that there are elevated circulatory levels of miR-128 in patients with T2D and depression [26]. Moreover, the miR-128a rs11888095 was reported to be associated with diabetic neuropathy in the Italian population. In the present study we examined the association of VEGF rs699947 C/A (-2578), VEGF-2549 insertion/deletion (I/D), ACE I/D rs4646994 and Mir128a (rs11888095) gene variations with T2D in subjects from KSA.

2. Material Methods

2.1. Study Population, Inclusion and Exclusion Criteria

This project was approved by the Research and Studies Department, Directorate of Health Affairs, Taif, approval No. 229, and by the Research Ethics Committee of the Armed Forces hospitals, Northwestern Region approval No. R & RE C2016-115. The population comprised T2D patients visiting the hospitals for routine checkup. The study included patients with clinically confirmed cases of T2D. The study included only citizens of Saudi Arabia, both males and females. All subjects gave informed consent. A standard questionnaire was used to document the socio-demographical characteristics such as age, sex and lifestyle.

The study excluded patients with T1D and patients with any previous history of any chronic diseases. The healthy controls (HCs) ranged from 20 to 80 years of age, and were visiting the hospital for a routine checkup. The controls were enrolled from the general population of the same geographical region. A routine medical check-up was conducted (CBC, KFT, LFT, etc.) and the history of illness, if detected, was recorded by a health practitioner. Those who appeared apparently healthy without any history of any significant
disease, or other chronic diseases, were considered normal. Figure 1 summarizes the procedure of study from samples collection to the statistical analyses.

Figure 1. Summary of the study explaining the procedure from sample collection until statistical analyses. Abbreviations. HCs: healthy controls. ARMS-PCR: amplification mutation system PCR. MSP: mutation specific PCR.

2.2. Sample Collection and Genomic DNA Extraction

All patient specimens were timed around the routine drawing of blood that was the part of a routine workout, and hence did not require additional phlebotomy. About 3 mL of peripheral blood was collected by venipuncture in EDTA tubes from T2D patients and from HCs. DNA was extracted using a DNeasy Blood Kit (Cat No. 69506) Qiagen (Hilden, Germany) as per the manufacturer’s instructions, then the DNA was dissolved in nuclease-free water and stored at 4 °C until use. The extracted DNA was dissolved in nuclease-free H2O and stored at 4 °C until use. The quality of the extracted DNA was checked by running the sample in 0.8% agarose gel. The quantity of the extracted DNA was determined by NanoDrop™ (Thermo Scientific, Waltham, MA, USA).

2.3. Genotyping of VEGF rs699947C/A and miR-128a rs11888095 C/T by Amplification Refractory Mutation System PCR (ARMS-PCR)

VEGF promoter region rs699947 C/A and miR-128 rs11888095 C > T genotyping was conducted by the ARMS-PCR [27,28]. The primers were designed using Primer3 software (Table 1). The ARMS-PCR was performed in a reaction volume of 25 µL containing template DNA (50 ng), Fo-0.25 µL, Ro-0.25 µL, FI-0.25 µL and RI-0.25 µL of 25 pmol of each primer, and 10 µL from GoTaq Green PCR Master Mix (2X) (Promega, Madison, WI, USA). The final volume of 25 µL was adjusted by adding nuclease-free ddH2O. Then, 2 µL of DNA was added from each subject.

Table 1. Genotyping primers of gene polymorphisms.

| Primers for VEGF-rs699947 (−2578) C/A Gene Polymorphism | Band | Band Size | A.Tm |
|----------------------------------------------------------|------|-----------|------|
| VEGF Fo 5-CCTTTTCTCATAAGGGGCTTAG-3 | Control band | 353 bp | 58 °C |
| VEGF Ro 5-AGGAAGCAGCTTGGAATAACC-3 | | | |
| VEGF FI A 5-TAGGCCAGACCTTGCAAA-3 | A-allele | 149bp | |
| VEGF RI C 5-GTCTGATTATCCACCCAGATCG-3 | C-allele | 243bp | |
Table 1. Cont.

| Primers for VEGF-rs699947 (−2578) C/A Gene Polymorphism | Band | Band Size | A.Tm |
|----------------------------------------------------------|------|-----------|------|
| ARMS primers for miR128a rs11888095 C/T                 |      |           |      |
| miR128 Fo                                               | 5'-AGATGGAATTTTACTGTGGTCTGT-3 | Control band | 441 bp | 55 °C |
| miR128 Ro                                               | 5-GCCAATTATTGCAAATATTAAATGTATAGG-3 |               |      |      |
| miR128 Fi                                               | 5'-ATGTATGCTTTGAAATGTGAAGGAT-3 | T-allele | 202 bp |      |
| miR128 RI                                               | 5-ATACTATACCACTCCCTATATGCATTG-3 | C-allele | 295 bp |      |

Primers for VEGF−2549 insertion/deletion (I/D) gene polymorphism

| VEGF F | 5'-GCTGAGAGTGGGGCTGACTAGGT-3' | D-allele | 211 bp | 58.8 °C |
| VEGF R | 5'-GTTTCTGACCTGGCTATTTCCAGG-3' | I-allele | 229 bp |      |

Primer sequence of ACE I/D rs4646994

| ACE F | 5'-GTGGAGACCACTCCCATCCTTTCT -3' | D | 190bp | 58 °C |
| ACE R | 5'-GATGTGGCCATCAACTTCGTCACGAT -3' | I | 490bp |      |

2.3.1. ARMS-PCR Programming

The PCR conditions optimized for VEGF (rs699947C/A) and miR128 rs11888095 C/T were with an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 35 s; annealing Tm was 58 °C for VEGF rs699947 and 55 °C for miR128a rs11888095 for 40 s, extension at 72 °C for 45 s, and a final extension step at 72 °C for 10 min.

2.3.2. Gel Electrophoresis for ARMS-PCR Products

The VEGF rs699947 (−2578 C/A) amplification products were separated by electrophoresis. Primers Fo and Ro flank the exon of the VEGF-2578 C/A gene, resulting in a band of 353 bp to act as a control for DNA quality and quantity. Primers FI and Ro amplify a wild-type allele (C allele), generating a band of 229 bp, and primers Fo and RI generate a band of 149 bp from the mutant allele (A allele) Figure 2.

![Figure 2](image)

Figure 2. Genotyping of the VEGF rs699947 C/A using ARMS-PCR in T2D subjects.

The mir128 rs11888095 C/T amplification products were separated by electrophoresis. Primers Fo and Ro of mir128 rs11888095 C/T resulted in a band of 458 bp to act as a control for DNA quality and quantity. Primers Fo and Ro amplify a wild-type allele (T allele), generating a band of 202 bp, and primers Fo and RI generate a band of 295 bp from the mutant allele (A allele) Figure 3.
2.4. Genotyping of VEGF I/D and ACE I/D rs4646994 by Mutation Specific PCR (MSP)

VEGF I/D and ACE I/D rs4646994 polymorphisms were genotyped using the MSP, with primers used by Amle et al. [29] for VEGF D/I. The primers were designed using the Primer3 software (Table 1). The PCR was undertaken in a reaction volume of 25 µL containing template DNA (50 ng), F-0. 25µL and R-0. 25 µL of 25 pmol of each primer, and 10 µL from GoTaq® Green Master Mix (cat no. M7122) (Promega, Madison, WI, USA). The final volume of 25 µL was adjusted by adding nuclease-free double distilled water (ddH₂O).

2.4.1. MSP Programming

The PCR conditions used were initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 35 s, annealing Tm for ACE I/D (58 °C) for 40 s and for VEGF I/D (58.80 °C) for 1 min, 72 °C for 45 s followed by the final extension at 72 °C for 10 min.

2.4.2. Gel Electrophoresis for MSP Products

The MSP product of the VEGF-2549 I/D products were separated on 1.5% agarose. There were two bands of 211 bp for the D allele and 229 bp for the I allele (Figure 4). The MSP product of ACE-I/D was separated on 1.5% agarose gel. There were 3 bands. The II genotype yielded a 490 bp fragment, the DD genotype yielded a 190 bp fragment, and ID yielded both 490 and 190 bp fragments (Figure 5).
2.5. Statistical Analysis

Group differences were compared using Student’s two-sample t-test or one-way analysis of variance (ANOVA) for continuous variables and chi-squared test for categorical variables. Deviations from the Hardy–Weinberg disequilibrium (HWD) were calculated by the chi-square (χ²) goodness-of-fit test. The differences in the VEGF rs699947 C/A, VEGF-2549 I/D, miR128 rs11888095 C/T and ACE I/D rs4646994 genotype frequencies between cases and controls were evaluated using the chi-square test. Associations between alleles and genotypes and the incidence of T2D were estimated with the odds ratios (ORs), and the risk ratios (RRs). We calculated the risk differences (RDs) with 95% confidence intervals (CIs). A p-value < 0.05 suggested a significant difference. Statistical analyses were performed using Graph Pad Prism 6.0 or SPSS 16.

3. Results

3.1. Genotypes Distribution of the Gene Polymorphisms

Our results showed that the VEGF rs699947 C/A genotype distribution was significantly different between the cases (34.4, 57.4 and 8.2%) and the healthy controls (46, 38.1 and 15.9%) with a p-value = 0.007 (Table 2). The results also indicated that the distribution of the VEGF I/D polymorphism was significantly different between the cases (15, 53.4 and 31.6), and the controls (22.6, 57.9 and 19.5) with a p-value= 0.049 (Table 3). Moreover, results indicated that there was significant difference in the genotype distribution of the ACE I/D between the cases (57.2, 36.2 and 6.6%) and controls (12, 40 and 48%) with p-value = 0.049 (Table 4). The genotype distribution of the mir128 rs11888095 C/T was also significantly different between cases (27, 53 and 20%) and the controls (55, 34 and 11%) with a p-value = 0.0001 (Table 5).

Table 2. The distribution of VEGF rs699947 C/A genotypes in T2D cases and controls.

| Subjects   | n  | CC | %   | CA | %   | AA | %   | Df | X²  | C  | A  | p-Value |
|------------|----|----|-----|----|-----|----|-----|-----|-----|-----|-----|---------|
| Cases      | 122| 42 | 34.4| 70 | 57.4| 10 | 8.2 | 2   | 9.93| 0.63| 0.37| 0.007   |
| Controls   | 126| 58 | 46  | 48 | 38.1| 20 | 15.9|     |     |     |     |         |

Table 3. The distribution of VEGF-2549 I/D polymorphism genotypes in T2D cases and controls.

| Subjects   | n  | I  | %   | ID | %   | D  | %   | Df | X²  | I  | D  | p-Value |
|------------|----|----|-----|----|-----|----|-----|-----|-----|-----|-----|---------|
| Cases      | 133| 20 | 15  | 71 | 53.4| 42 | 31.6| 2   | 6   | 0.42| 0.58| 0.049   |
| Controls   | 133| 30 | 22.6| 77 | 57.9| 26 | 19.5|     |     |     |     |         |
Table 4. Genotype distribution of ACE I/D rs4646994 between T2D cases and controls.

| Subjects | n   | II %   | ID %   | DD %   | I    | D    | DF   | X2   | p-Value |
|----------|-----|--------|--------|--------|------|------|------|------|---------|
| Cases    | 152 | 87 (57.23%) | 55 (36.2%) | 10 (6.57%) | 0.75 | 0.25 | 2    | 92.43 | <0.0001 |
| Controls | 150 | 18 (12%)   | 60 (40%) | 72 (48%)  | 0.32 | 0.68 |      |      |         |

Table 5. Genotype distribution mir128 rs11888095 C/T genotypes in T2D cases and controls.

| Subjects | n   | CC   | CT   | TT   | T | C | Df | X2   | p-Value |
|----------|-----|------|------|------|--|--|--|--|---------|
| Cases    | 129 | 35 (27.13%) | 68 (52.71%) | 26 (20.16%) | 0.54 | 0.46 | 2 | 20.06 | 0.0001 |
| Controls | 112 | 62 (55.35%) | 38 (33.92%) | 12 (10.70%) | 0.73 | 0.27 | |        |         |

3.2. The Association of the VEGF rs699947 C/A SNP with T2D

The results indicated that VEGF rs699947 C/A SNP was associated with T2D, the CA genotype was associated with T2D with OR (95% CI) = 2.01 (1.17–3.45), RR = 1.42 (1.08–1.87), p-value = 0.011 (Table 6). Our results showed that there was a significant difference (p-value < 0.05) in the VEGF rs699947 C/A genotype distribution between male and female cases, and between cases >25 years and cases >40 years (Table 7). The results also showed that there were significant differences (p-values < 0.05) between different genotypes of the VEGF rs699947 in the patients with a normal lipid profile and cases with an abnormal lipid profile (Table 7).

Table 6. Association of VEGF rs699947 C/A SNP with T2D.

| Genotypes       | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|-----------------|------------------|-----------|-------------|----------------|--|
|                 | (n = 126)        | (n = 122) |             |                |         |
| Codominant      |                  |           |             |                |         |
| VEGF–(C)        | 58               | 46        | 42          | 34.4           | 1 (ref.)| 1 (ref.)|      |         |
| VEGF-(CA)       | 48               | 38.1      | 70          | 57.4           | 2.01 (1.17–3.45) | 1.42 (1.08–1.87) | 0.011 * |         |
| VEGF–(A)        | 20               | 15.9      | 10          | 8.2            | 0.69 (0.29–1.62) | 0.87 (0.64–1.17) | 0.363 |         |
| Dominant        |                  |           |             |                |         |
| VEGF (C)        | 58               | 46        | 42          | 34.4           | 1 (ref.)| 1 (ref.)|      |         |
| VEGF-(CA + A)   | 68               | 54        | 80          | 65.6           | 1.62 (0.97–2.71) | 1.26 (0.99–1.60) | 0.05 |         |
| Recessive       |                  |           |             |                |         |
| VEGF-(C + CA)   | 106              | 84        | 112         | 91.8           | 1 (ref.)| 1 (ref.)|      |         |
| VEGF–(A)        | 20               | 16        | 10          | 8.2            | 0.47 (0.211–1.05) | 0.72 (0.54–0.97) | 0.06 |         |
| Allele          |                  |           |             |                |         |
| VEGF-(C)        | 164              | 154       | 1           | 1.08           | 1.04 (0.86–1.25) | 0.64 |         |
| VEGF–(A)        | 88               | 90        |             |                |         |

* Statistically significant difference.

Table 7. Association of VEGF rs699947 C/A genotypes with T2D patients’ characteristics.

| Clinical Feature | CC 42 | CA 70 | AA 10 | X2  | DF  | p-Value |
|------------------|-------|-------|-------|-----|-----|---------|
| Gender           |       |       |       |     |     |         |
| Male             | 82    | 18 (14.75%) | 58 (47.54%) | 6 (4.91%) | 19.32 | 2 | 0.0001 * |
| Female           | 40    | 24 (19.67%) | 12 (9.83%)  | 4 (3.27%)  |     |     |         |
Table 7. Cont.

| Clinical Feature | CC 42 | CA 70 | AA 10 | X2 | DF | p-Value |
|------------------|-------|-------|-------|----|----|---------|
| Age              |       |       |       |    |    |         |
| >40              | 91    | 36 (29.50%) | 50 (40.98%) | 5 (4.09%) | 6.3 | 2 | 0.042 * |
| >25              | 31    | 6 (4.8%) | 20 (9.6%) | 5 (1.9%) |    |    |         |
| HbA1c%           |       |       |       |    |    |         |
| >6               | 102   | 36 (29.50%) | 60 (49.18%) | 6 (4.91%) | 4.43 | 2 | 0.100 |
| <6               | 20    | 6 (4.91%) | 10 (8.19%) | 4 (3.27%) |    |    |         |
| TG mg/dl         |       |       |       |    |    |         |
| <200             | 49    | 25 (51%) | 20 (40.81%) | 4 (8.16%) | 10.16 | 2 | 0.005 * |
| >200             | 73    | 17 (23.28%) | 50 (68.49%) | 6 (8.21%) |    |    |         |
| TC mg/dl         |       |       |       |    |    |         |
| <200             | 64    | 12 (18.75%) | 45 (70.31%) | 7 (10.93%) | 14.12 | 2 | 0.006 * |
| >200             | 58    | 30 (51.72%) | 25 (43.10%) | 3 (5.17%) |    |    |         |
| LDL-C mg/dl      |       |       |       |    |    |         |
| <100             | 66    | 32 (57.57%) | 30 (45.45%) | 4 (6.06%) | 12.6 | 2 | 0.0018 * |
| >100             | 56    | 10 (10.85%) | 40 (71.42%) | 6 (10.71%) |    |    |         |
| HDL-C mg/dl      |       |       |       |    |    |         |
| <55              | 49    | 9 (18.36%) | 34 (69.38%) | 6 (12.24%) | 8.32 | 2 | 0.015 * |
| >55              | 73    | 31 (42.46%) | 38 (52%) | 4 (5.47%) |    |    |         |
| VITD ng/mL       |       |       |       |    |    |         |
| <30              | 16    | 6 (5.8%) | 10 (9.6%) | 0 (0%) | 1.343 | 4 | 0.854 |
| >30              | 14    | 5 (4.8%) | 8 (7.7%) | 1 (1%) |    |    |         |

The association of VEGF rs699947 C/A genotypes with T2D patients characteristics. The gender, age, HbA1c%, cholesterol mg/dl, LDL-C mg/dl and HDL-C mg/dl are based on data for 122 cases. For the vitamin D ng/mL (VITD), data was collected for 30 cases. * Statistically significant difference.

3.3. The Association of VEGF-2549 I/D Polymorphism with T2D

Results indicated that the D allele of the VEGF I/D at the –2549 position was associated with T2D with OR (95% CI) = 1.43 (1.02–2.03), RR = 1.01 (0.83–1.21), p-value = 0.037 (Table 8). The results also showed that there were significant differences (p-values < 0.05) between different genotypes of the VEGF-2549 I/D polymorphism in patients with a normal lipid profile and patients with an abnormal lipid profile (Table 9).

Table 8. Association of VEGF-2549 I/D polymorphism with T2D.

| Genotypes | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|-----------|------------------|-----------|-------------|----------------|---------|
| (n = 133) | %                | (n = 133) | %           |                |         |
| Codominant|                  |           |             |                |         |
| VEGF–(I)  | 30               | 22.55     | 20          | 15.03          | 1 (ref.) | 1 (ref.) |
| VEGF-(ID) | 77               | 57.89     | 71          | 53.38          | 1.38 (0.72–2.65) | 1.15 (0.87–1.51) | 0.32 |
| VEGF–(D)  | 26               | 19.54     | 42          | 31.57          | 2.42 (1.14–5.11) | 1.56 (1.07–2.28) | 0.010 * |
| Dominant  |                  |           |             |                |         |

...
Table 8. Cont.

| Genotypes     | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|---------------|------------------|-----------|-------------|----------------|---------|
| VEGF-(I)      | 30               | 22.55     | 15.03       | 1 (ref.)       | 1 (ref.)|
| VEGF-(ID + D) | 103              | 77.44     | 84.96       | 1.64 (0.88–3.07)| 1.25 (0.96–1.64) | 0.090 |
| Recessive     |                  |           |             |                |         |
| VEGF-(I + ID) | 107              | 80.45     | 68.42       | 1 (ref.)       | 1 (ref.)|
| VEGF-(D)      | 26               | 19.54     | 31.57       | 1.89 (1.08–3.33)| 1.37 (0.98–1.91) | 0.025 *|
| Allele        |                  |           |             |                |         |
| VEGF-(I)      | 133              | 100       | 111         | 83.45          | 1 (ref.)|
| VEGF-(D)      | 129              | 97        | 155         | 116.54         | 1.43 (1.02–2.03) | 1.01 (0.83–1.21) | 0.037 *|

* Statistically significant difference.

Table 9. Association of VEGF-2549 I/D genotypes with T2D patients’ characteristics.

| Clinical Feature | n   | D %  | I %  | ID % | X2   | DF  | p-Value |
|------------------|-----|------|------|------|------|-----|---------|
| Gender           |     |      |      |      |      |     |         |
| Female           | 40  | 7 (6.5%) | 9 (8.4%) | 24 (22.4%) | 1.498 | 2   | 0.473   |
| Male             | 67  | 14 (13.1%) | 9 (8.4%) | 44 (41.1%) |      |     |         |
| Age              |     |      |      |      |      |     |         |
| >25              | 16  | 4 (3.7%) | 1 (0.9%) | 11 (10.3%) | 1.607 | 2   | 0.448   |
| >40              | 91  | 17 (15.9%) | 17 (15.9%) | 57 (53.3%) |      |     |         |
| HbA1c%           |     |      |      |      |      |     |         |
| <6               | 1   | 0 (0.0%) | 0 (0.0%) | 1 (0.9%) | 0.579 | 2   | 0.749   |
| >6               | 96  | 21 (19.6%) | 18 (16.8%) | 67 (62.6%) |      |     |         |
| TC mg/dl         |     |      |      |      |      |     |         |
| <200             | 65  | 12 (11.2%) | 7 (6.5%) | 46 (43.0%) | 11.411 | 4   | 0.022 *|
| >200             | 17  | 6 (35.29%) | 2 (11.76%) | 9 (52.94%) |      |     |         |
| TG mg/dl         |     |      |      |      |      |     |         |
| <200             | 65  | 12 (18.46%) | 7 (10.76%) | 46 (70.76%) | 11.0 | 2   | 0.004 *|
| >200             | 31  | 12 (38.70%) | 8 (16.12%) | 11 (35.48%) |      |     |         |
| LDL-C mg/dl      |     |      |      |      |      |     |         |
| <100             | 34  | 4 (3.7%) | 2 (1.9%) | 26 (24.3%) | 11.364 | 4   | 0.023 *|
| >100             | 51  | 14 (13.1%) | 8 (7.5%) | 29 (27.1%) |      |     |         |
| HDL-C mg/dl      |     |      |      |      |      |     |         |
| <55              | 26  | 17 (15.9%) | 9 (8.4%) | 45 (42.1%) | 13.106 | 4   | 0.011 *|
| >55              | 12  | 1 (0.9%) | 0 (0.0%) | 11 (10.3%) |      |     |         |
| VIT D ng/ml      |     |      |      |      |      |     |         |
| <30              | 22  | 3 (2.8%) | 2 (1.9%) | 17 (15.9%) | 2.777 | 4   | 0.596   |
| >30              | 15  | 3 (2.8%) | 2 (1.9%) | 10 (9.3%) |      |     |         |

* Statistically significant difference. Association of VEGF-2549 I/D Genotypes with T2D Patient’s Characteristics.

The gender and age are based on data for 107 cases. HbA1c% is based on data for 97 cases, cholesterol mg/dl—82 cases, LDL-C mg/dl—85 cases, HDL-C mg/dl—38 cases, and vitamin D ng/mL (VIT.D)—37 cases.
3.4. Association of ACE I/D rs4646994 Polymorphism with T2D Patients

Results showed that the ACE I/D polymorphism was associated with T2D (Table 10). The ACE–ID genotype was associated with T2D with OR = 0.18 (0.101–0.354), RR = 0.32 (0.208–0.518), p-value = 0.0001 (Table 10). The ACE–DD genotype was also associated with T2D with OR = 0.128 (0.0125–0.0661), RR = 0.19 (0.1272–0.2996), p-value = 0.0001 (Table 10). The D allele was associated with T2D with OR = 0.10 (0.12–0.2), RR = 0.40 (0.3–0.5), p-value = 0.0001 (Table 10). Our results indicated that there were a significant different in ACE I/D genotype distribution between cases aged >25 years and cases aged <25 years (Table 11). The results showed that there was a significant difference (p-value = 0.02) in the ACE I/D genotype distribution between cases with normal and cases with elevated HbA1c (Table 11). Moreover, results showed that there were significant differences (p-values < 0.05) in the ACE I/D genotype distribution in cases with normal and abnormal lipid profiles (Table 11).

Table 10. Association of ACE I/D polymorphism with T2D.

| Genotypes          | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|--------------------|------------------|-----------|-------------|-----------------|---------|
| (n = 150)          | (n = 152)        |           |             |                 |         |
| Co-dominant        |                  |           |             |                 |         |
| ACE–II             | 18               | 87        | 1 (ref.)    | 1 (ref.)        |         |
| ACE–ID             | 60               | 55        | 0.18 (0.1–0.35) | 0.32 (0.208–0.518) | 0.0001 * |
| ACE–DD             | 72               | 10        | 0.128 (0.01–0.1) | 0.19 (0.1272–0.2996) | <0.0001 * |
| Dominant           |                  |           |             |                 |         |
| ACE–II             | 18               | 87        | 1 (ref.)    | 1 (ref.)        |         |
| ACE–(DI+DD)        | 132              | 65        | 0.10 (0.06–0.18) | 0.25 (0.1661–0.3940) | <0.0001 * |
| Recessive          |                  |           |             |                 |         |
| ACE–(II+DI)        | 78               | 142       | 1 (ref.)    | 1 (ref.)        |         |
| ACE–DD             | 72               | 10        | 0.076 (0.04–0.15) | 0.40 (0.3–0.49) | <0.0001 * |
| Allele             |                  |           |             |                 |         |
| ACE–I              | 96               | 229       | 1 (ref.)    | 1 (ref.)        |         |
| ACE–D              | 204              | 75        | 0.10 (0.1–0.2) | 0.40 (0.33–0.48) | 0.0001 * |
| Over dominant      |                  |           |             |                 |         |
| ACE–II+DD          | 90               | 97        | 1 (ref.)    | 1 (ref.)        |         |
| ACE–ID             | 60               | 55        | 0.85 (0.5–1.4) | 0.92 (0.73–1.16) | 0.4910  |

* Statistically significant difference.

Table 11. Association of ACE I/D polymorphism genotype with patients’ characteristics.

| Clinical Feature | n   | n = 152 | II  | DI  | DD  | X2  | DF  | p-Value |
|------------------|-----|---------|-----|-----|-----|-----|-----|---------|
| Gender           |     |         |     |     |     |     |     |         |
| Male             | 44  | 25      | 17  | 02  |     | 0.49| 2   | 0.78    |
| Female           | 108 | 62      | 38  | 08  |     |     |     |         |
| Age              |     |         |     |     |     |     |     |         |
| >25              | 96  | 41      | 46  | 09  |     | 22.62| 2 | 0.0001 * |
| <25              | 56  | 46      | 09  | 01  |     |     |     |         |
Table 11. Cont.

| Clinical Feature | n = 152 | II | DI | DD | X2 | DF | p-Value |
|------------------|---------|----|----|----|----|----|---------|
| HBA1c%           |         |    |    |    |    |    |         |
| >6               | 103     | 52 | 45 | 06 | 7.79 | 2 | 0.020 * |
| <6               | 49      | 35 | 10 | 04 |       |   |         |
| TG mg/dl         |         |    |    |    |    |    |         |
| <200             | 80      | 46 | 25 | 09 | 6.74 | 2 | 0.0344 *|
| >200             | 72      | 41 | 30 | 01 |       |   |         |
| TC mg/dl         |         |    |    |    |    |    |         |
| <200             | 99      | 50 | 43 | 06 | 6.49 | 2 | 0.039 * |
| >200             | 53      | 37 | 12 | 4 |       |   |         |
| LDL-C mg/dl      |         |    |    |    |    |    |         |
| <100             | 82      | 37 | 30 | 01 | 7.19 | 2 | 0.022 * |
| >100             | 70      | 50 | 25 | 09 |       |   |         |
| HDL-C mg/dl      |         |    |    |    |    |    |         |
| <55              | 104     | 58 | 42 | 04 | 5.47 | 2 | 0.64    |
| >55              | 48      | 29 | 13 | 06 |       |   |         |

* Statistically significant difference.

3.5. Association of miR128 rs11888095 C/T SNP with T2D Patients

Results showed that the CT genotype of the miR128 rs11888095 was associated with T2D with OR = 3.16 (1.8–5.6), RR = 1.78 (1.3–2.4), p-value = 0.0001 (Table 12). The results also indicated that the T allele was associated with T2D with OR = 1.68 (1.13–2.5), RR = 1.36 (1.069–1.7400), p-value = 0.0105 (Table 12). Our results showed that there was a significant difference (p-value = 0.031) in the miR128 rs11888095 genotype distribution between male and female cases (Table 13). Moreover, the results showed that there was a significant difference (p-value = 0.021) in cases aged <25 years and cases aged >25 years (Table 13). Moreover, results showed that there a significant difference (p-values < 0.05) in cases with normal and cases with elevated HbA1c (Table 13). Furthermore, results showed that there was a significant difference (p-values < 0.05) in the genotype distribution of cases with normal and cases with elevated lipid profiles (Table 13).

Table 12. Association of miR128 rs11888095 C/T with T2D.

| Genotypes       | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|-----------------|------------------|-----------|-------------|-----------------|---------|
| Codominant      |                  |           |             |                 |         |
| miR128 –(C)     | 62               | 35        | Ref         | Ref             |         |
| miR128-(CT)     | 38               | 68        | 3.16(1.8–5.6)| 1.78(1.3–2.4)   | 0.0001 *|
| miR128-(T)      | 12               | 26        | 3.83(1.7–8.5)| 2.0 (1.2–3.3)   | 0.0010 *|
| Dominant        |                  |           |             |                 |         |
| miR128-(C)      | 62               | 35        | Ref         | Ref             |         |
| miR128-(CT + T) | 50               | 94        | 3.3(1.9–5.7) | 1.84(1.4063–2.4097)| <0.0001 *|
| miR128-(T)      | 12               | 26        | 3.3(1.9–5.7) | 1.55(0.96–2.5)  | <0.0001 *|
Table 12. Cont.

| Genotypes | Healthy Controls | T2D Cases | OR (95% CI) | Risk Ratio (RR) | p-Value |
|-----------|-----------------|-----------|-------------|-----------------|---------|
| Allele    |                 |           |             |                 |         |
| miR128-(C) | 112             | 129       | Ref         | Ref             |         |
| miR128-(T) | 62              | 120       | 1.68(1.13–2.5) | 1.36 (1.1–1.7) | 0.0105 * |

* Statistically significant difference.

Table 13. Association of miR128 rs11888095 C/T genotypes with T2D patients’ characteristics.

| Clinical Feature | n   | CC | CT | TT | X2  | DF | p-Value |
|------------------|-----|----|----|----|-----|----|---------|
| Gender           | 129 | 35 | 68 | 26 |     |    |         |
|                  |     |    |    |    |     |    |         |
| Male             | 40  | 7  | 28 | 05 | 6.95| 2  | 0.031 * |
| Female           | 89  | 28 | 40 | 21 |     |    |         |
| Age              |     |    |    |    |     |    |         |
| >25              | 109 | 26 | 59 | 25 | 7.53| 2  | 0.021 * |
| <25              | 20  | 10 | 09 | 01 |     |    |         |
| HBA1c%           |     |    |    |    |     |    |         |
| >6               | 82  | 12 | 49 | 21 | 18  | 2  | 0.0001 *|
| <6               | 47  | 23 | 19 | 05 |     |    |         |
| TG mg/dl         |     |    |    |    |     |    |         |
| <200             | 82  | 14 | 48 | 20 | 11.84| 2  | 0.0027 *|
| >200             | 47  | 21 | 20 | 06 |     |    |         |
| TC mg/dl         |     |    |    |    |     |    |         |
| <200             | 85  | 28 | 40 | 17 | 0.52 | 2  | 0.776  |
| >200             | 44  | 17 | 18 | 09 |     |    |         |
| LDL-C mg/dl      |     |    |    |    |     |    |         |
| <100             | 56  | 08 | 34 | 14 | 8.37 | 2  | 0.0152 *|
| >100             | 73  | 27 | 34 | 12 |     |    |         |
| HDL-C mg/dl      |     |    |    |    |     |    |         |
| <55              | 98  | 25 | 54 | 19 | 0.96 | 2  | 0.6188 |
| >55              | 31  | 10 | 14 | 07 |     |    |         |

* Statistically significant difference.

4. Discussion

T2D is developed by a mixture of insulin resistance and impaired secretion of insulin [6]. The VEGF stimulates the cell proliferation, migration and vasopermeability in many tissues [30]. It has been reported that there is association between the VEGF expression and metabolic syndrome [31]. Metabolic syndrome is defined as insulin resistance associated with obesity, hypertension and dyslipidemia [31]. It has been suggested that increased levels of VEGF are associated with increased blood sugar [31]. Our results showed that there was a significant difference (p-value = 0.007) in the genotype distribution of VEGF rs699947 C/A between the cases and the controls (Table 2). The CA genotype was also associated with T2D (Table 6).

Results showed that there was a significant difference (p-values < 0.05) in the VEGF rs699947 genotype distribution between cases with normal and cases with abnormal lipid profiles (Table 7). This result is consistent with studies that reported the association of
VEGF rs699947 with the risk of cardiovascular disease [32,33]. The results also showed that males with the rs699947 CA genotype and A allele are more susceptible to T2D than females (p-values < 0.05, Table 7). This result is consistent with studies reporting higher prevalence of T2D among males than females [34,35]. Our results also indicated that elder individuals (age > 40) with the CA genotype are more prone to T2D than younger individuals (<40 years old, p-values < 0.05, Table 6). This is also in agreement with a previous study that reported that T2D is more prevalent in older individuals in all populations [36]. We did not observe significant differences in the VEGF rs699947 genotype distribution in the cases with normal or elevated HbA1c (p-values > 0.05, Table 7). This is probably because of the relatively small sample size used in this study. This may be one of the limitations of this study.

The VEGF gene I/D polymorphism is an 18 bp fragment found at the −2549 position of the promoter region [29]. The results showed that there is a significant difference (p-value < 0.05) in the genotype distribution of VEGF I/D polymorphism between the cases and healthy controls (Table 3). The results indicated that the D allele of the VEGF I/D was associated with T2D (Table 8). Moreover, results indicated that there was a significant difference (p-values < 0.05) between genotypes of the VEGF I/D polymorphism in the patients with normal and patients with abnormal lipid profiles (Table 9). Gene variations in the VEGF gene promoter such as I/D polymorphism were reported to increase expression of the VEGF [30,37]. Therefore, these results may be in agreement with the result of Zafar et al., who reported that the increased expression of the VEGF gene has been associated with metabolic syndromes, such as hypertriglyceridemia [31]. Several previous studies have implicated the associations of diabetes complications with gene variations at the VEGF [38]. For instance, the VEGF gene at position −7°C/T was reported to be associated with diabetic neuropathy in a population of British Caucasians [39]. In addition, the −634 G/C SNP in the 5′UTR of the VEGF gene was associated with risk diabetic retinopathy (DR) in the Japanese population [40]. Furthermore, Buraczynska et al. showed that the VEGF I/D polymorphism was associated with risk of DR in the Polish population [37]. Churchill et al. reported the association of the VEGF promoter SNPs rs735286 and rs2146323 with the severity of DR in the British population [41], whereas Han et al. reported the association of VEGF gene SNPs rs3025039 and rs833061 with DR in the Chinese population [42]. Furthermore, the −2578 C/A, rs699947, was associated with diabetic foot ulcers in Iranian and Chinese Han populations [43,44]. All these studies may be in agreement with our results as they report the association of VEGF gene variations with diabetes complications, and our results showed that the VEGF gene variations rs699947 and VEGF I/D polymorphisms were associated with the risk of T2D (Tables 6 and 8). Furthermore, it has been reported that the gene variations in the VEGF promoter (e.g., VEGF rs699947 and VEGF I/D) result in enhanced expression of the VEGF gene [30,37]. The increased expression of the VEGF gene was associated with insulin resistance [31], and that the neutralization of the VEGF gene resulted in improvement in insulin sensitivity in the liver and in fat tissues [45]. These studies may be in agreement with our results, which showed that VEGF rs699947 and VEGF I/D are associated with T2D risk.

Our results indicated that there was a significant difference in the ACE I/D polymorphism genotype distribution between cases and controls (p-value < 0.05, Table 4). The ACE I/D polymorphism was associated with T2D (Table 10). The results indicated that the ACE ID genotype and the ACE D allele were associated with decreased risk of T2D (Table 10). This result may be partially consistent with the results of Al-Serri et al. (2015) [46], who reported that the I allele of the ACE I/D polymorphism is associated with T2D in the Kuwaiti population. However, our result is in disagreement with the study of Al-Rubeaan et al. (2013) [47]. This disagreement may be due to different sample sizes or different populations. The results indicated that there was a significant difference in the ACE I/D polymorphism genotype distribution between males and females (Table 11). The results also showed that there were significant differences in the ACE I/D polymorphism genotype distribution between cases with elevated and cases with normal HbA1c, and
between cases with normal and cases with abnormal lipid profiles (Table 11). These results are perhaps in agreement with studies reporting the association of ACE I/D polymorphism with dyslipidemia [48,49]. A previous study also proposed the ACE as a target for the protection of pancreatic beta cells from dysfunction causing T2D [50].

Results indicated the miR128 rs11888095 may be associated with development of T2D (Tables 5 and 12). The CT genotype and T allele of the miR128 rs11888095 were associated with risk of T2D (Table 12). This result is consistent with a study reporting that miR128 regulates genes (e.g., Insr, Irs1 and Pik3r1) critical for insulin signaling [51]. Our result is in partial agreement with a study indicating that the T allele of the miR128 rs11888095 is associated with diabetic polyneuropathy [52,53]. The results showed that females carrying the CT genotype and T allele of the miR128 rs11888095 are more susceptible to T2D than males carrying the CT genotype and T allele (Table 13). Furthermore, individuals with the CT genotype and T allele aged >25 years are more susceptible to T2D (Table 13). In addition, results showed that the CT genotype and T allele were more frequently present in cases with elevated HbA1c (>6%) than in cases with normal HbA1c (<6%) (Table 13). This result is expected because miR128 rs11888095 may be associated with T2D (Table 12). Moreover, it was indicated that miR128 rs11888095 may be associated with an elevated lipid profile (Table 13); this is quite consistent with a study reporting that mir128 regulates the gene involved in lipid metabolism [54]. Limitations of this study include the small sample size, and that it is a cross-sectional study, i.e., the samples may have been collected from cases after the blood chemistry was already maintained. Future longitudinal studies with larger sample sizes and in different populations are required. Because T2D can be delayed or prevented by diet modification, weight management, regular exercise and other factors [55,56], results of the present study can be used (after confirmation in future studies) in genetic testing and personalized advice to identify and stratify the individuals that are at risk of developing T2D.

5. Conclusions

We investigated the association of the VEGF promoter gene variations (VEGF rs699947, VEGF I/D), ACE I/D (rs4646994) and miR128 (rs11888095) with T2D in the Saudi population. The results showed that the CA genotype of the VEGF rs699947, the D allele of the VEGF I/D, and the TC genotype and T allele of the Mir128a (rs11888095) were associated with an increased risk of T2D. Moreover, the results indicated the D allele of the ACE I/D was protective against T2D. Further well-designed studies with larger sample sizes in different populations are required. Moreover, proteomics investigations [57–59] to examine the effects of SNPs on VEGF and ACE function are recommended.

Author Contributions: Conceptualization, I.E., R.M., F.M.A.D., M.A.A., A.I.A., J.B., A.T.B., M.M.M., F.A., H.M., E.A.M.F., methodology, R.M. and I.E.; validation, R.M. and I.E.; formal analysis, R.M. and I.E.; investigation, All authors; resources, All authors; data curation, R.M. and I.E.; writing—original draft preparation, R.M. and I.E.; writing—review and editing, I.E.; visualization, I.E., R.M., F.M.A.D., M.A.A., A.I.A., J.B., A.T.B., M.M.M., F.A., H.M., E.A.M.F.; supervision I.E.; funding acquisition, I.E., R.M., F.M.A.D., M.A.A., A.I.A., J.B., A.T.B., M.M.M., F.A., H.M., E.A.M.F. All authors have read and agreed to the published version of the manuscript.

Funding: This project is funded by the University of Tabuk, Deanship of scientific research (DSR), project No 0012-1441-S for IE and colleagues.

Institutional Review Board Statement: This study was approved by the research and studies department, directorate of health affairs, Taif, approval No. 229, and by the research ethics committee of the armed forces hospitals, northwestern region, Tabuk, approval No. R & REC2016-115.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.
Acknowledgments: We thank T2D patients and healthy controls for their participation in this study. This project is funded by the University of Tabuk, Deanship of scientific research (DSR), project No. 0012-1441-S for IE and colleagues.

Conflicts of Interest: The authors declare no conflict of interest.

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