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# *MOLECULAR BASED DETECTION OF A PIK3C3 (PHOSPHATIDYLINOSITOL 3-KINASE) GENE VARIATIONS IN T2D PATIENTS OF TABUK-SAUDI ARABIA .*

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**Abstract:**

**Background***: The PI3K pathway is commonly dysregulated in different diseases such as cancer, with the most common events being mutation or increased gene copy numbers of* PIK3CA *or other* PI3K *isoforms, loss of expression of the pathway suppressors (for example, PTEN), or hyperactivation of RTKs through receptor overexpression or activating mutations. Several gene polymorphisms have been frequently associated with the onset of T2D. Therefore the aim of this study was to optimize molecular based assay for the detection of PIK3C rs121913281 C>T gene variation in T2D patients.* **Methodology***: This study was conducted on 60 samples among which 30 were T2D patients and 30 were healthy controls. DNA was extracted by Qiagen Kit and AS-PCR system was optimized to detect PIK3C rs121913281 C>T gene variation in T2D patients.* **Results:** *This study was conducted on 60 samples among which 30 were T2D patients and 30 were healthy controls. The 30 newly diagnosed T2D patients. AS-PCR system was optimized to detect PIK3C rs121913281 C>T gene variation in clinically confirmed cases of T2D patients and healthy controls. The technique was successfully optimized by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known SNPS in PIK3C rs121913281 C>T. Result indicated that AS-PCR technique can be used as a potential molecular tool in the detection of potential PIK3C gene variations in T2Ds.***Conclusion***: Allele Specific-PCR for PIK3CA- rs121913281 C>T gene variation was successfully optimized. The assay proved to be fast, accurate, simple and economical that does not entail any special equipment other than a thermocycler and gel documentation system.*

***Key Words: Type 2 diabetes (T2D), Allele-specific PCR, PIK3CA, Phosphatidylinositol 3-kinases (PI3Ks), rs121913281 C>T***

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## INTRODUCTION:

The International Diabetes Federation has estimated that there are about 415 million people with diabetes mellitus in the world. This number can be increased to increase to 642 million by the year 2040[1]. This disorder accounts for high morbidity and mortality due to complications like renal failure, blindness, limps amputations and cardiovascular diseases [2]. The International Diabetes Federation has reported that if the Saudi lifestyle and habits are not modified, about 50% Saudis will be diabetic by the next 10 years[3]. The complex etiology of T2D includes genetic and environmental risk factors. Environmental risk factors include lifestyle, obesity, dyslipidemia, lack of exercise, poor diet[4]. Regarding genetics risk factors, the genome wide association studies (GWASs) have shown association of T2D with nearly 80 candidate genes [5-8].

Phosphatidylinositol constitutes an important component of the phospholipid found in the plasma membrane of eukaryotic cell and involved various intracellular signaling cascades [9]. Phosphate group is added by kinases to three of the free hydroxyls at the head group of the phosphatidylinositol. These phosphorylated phosphatidylinositol regulate various vital cellular processes such as glucose homeostasis, cell survival, cell proliferation, cytoskeletal organization and function of platelet, cancer cell growth [10]. The phosphatidylinositol 3-kinase (PI3K) is an important element in the insulin signaling pathway[11] PI3K is activated by insulin; G-protein coupled receptor (GPCR), or cytokine receptor to produce phosphatidylinositol-3, 4, 5-triphosphate (PIP3) from the phosphatidylinositol-4, 5-bisphosphate (PIP2)[12]. The PIP3 is a key molecule of many cellular processes[12]. PI3K is a heterodimeric enzyme composed of a catalytic subunit (p110) and a regulatory subunit (p85) [13]. Impairment of insulin signaling pathway is associated with various diseases such as obesity, T2D, inflammation and cardiovascular diseases[14]. T2D is mainly characterized by an insufficient amount of insulin secreted by the pancreatic beta cells as well as impaired insulin action [15]. Insulin resistance is defined as a condition in which the insulin-stimulated uptake of glucose is failed in insulin sensitive tissues. This failure is due to the impairment of insulin signaling pathway[16]. The hyperglycemia is resulted when the beta cells secretion of insulin is unable to overcome the insulin resistance. Heterozygous mutation (R649W) in p85α regulatory subunit of PI3K has been demonstrated to influences the PI3K activity and its role in insulin action and metabolism of glucose[17]. In addition, mutation in PI3K has been suggested to be tumor marker and in future can be used for cancer treatment and diagnosis[18]. It is very important to develop efficient strategies to improve the prevention, and detection, and therapy of T2D in Saudi population through the screening susceptibility variants across the entire genome in an unbiased and effective manner. The aim of this study was to optimize molecular based assay for the detection of PIK3C rs121913281 C>T gene variation in T2D patients.

## MATERIALS AND METHODS:

**Study Population:** This study was done on 60 specimens among whom 30 were clinically confirmed cases of T2D patients and 30 healthy controls.

**Inclusion criteria:** The study included clinically confirmed cases of T2D patients and 30 healthy controls. All participants were of Saudi origin.

**Exclusion Criteria:** Patients with any other significant malignancy. Non Saudi Arabians were excluded.

**Sample collection:** After assessing the Laboratory findings, a 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from clinically confirmed cases of T2D patients and healthy controls in EDTA tubes after obtaining a written informed consent form.

**Sample Size:** The study was conducted on 60 specimens among whom 30 were clinically confirmed T2D patients and 30 were healthy controls.

**Genomic DNA extraction:** The DNA was isolated from T2D patients and healthy controls by using DNeasy Blood Kit (Qiagen) as per the manufactures instructions. The DNA extracted was dissolved in nuclease-free water, and stored at 4°C until use. The DNA quality and yield was assessed using Nanodrop (optical density) and 1.5% agarose gel electrophoresis

**Genotyping for PIK3CA- rs121913281 C>T**

PIK3CA- rs121913281 C>T gene variation detected by Allele specific-PCR approach .AS-PCR primers were designed by using Primer3 software as shown table 1.

| Table N0-1 : PIK3CA- rs121913281 C>T CAT ⇒ TAT H [19] ⇒ Y [20] | | | | |
|---|---|---|---|---|
| **Allele specific-PCR for C allele (Histine)** | | | | |
| PIK3CA-F1 | **C allele** | 5'-TTTCATGAAACAAATGAATGATGCAC-3' | **362 bp** | 57 $^0$C |
| PIK3CA-R | | 5'-TTCAAAGTTTACCTTTTTGGACTTAAGGC-3' | | |
| **Allele specific-PCR for T allele (Tyr )** | | | | |
| PIK3CA-F2 | **T allele** | 5'-TTTCATGAAACAAATGAATGATGCAT-3' | **362 bp** | 57 $^0$C |
| PIK3CA-R | | 5'-TTCAAAGTTTACCTTTTTGGACTTAAGGC-3' | | |

The AS-PCR for PIK3CA- rs121913281 C>T gene polymorphism was performed in a reaction volume of 25uL containing template DNA (50ng), The cocktail was prepared for 10 samples as depicted in table 3 F1-0.25uL , R -0.25uL, F2-0.25uL, R -0.25uL of 25pmol of each primers and 10uL from DreamTaq green Master Mix (Thermo , USA). The final volume of 25 uL was adjusted by adding nuclease free dd$H_2O$ .Finally 2ul of DNA was added from each T2D patient.

| Table No 2: Preparation of PCR cocktail for PIK3CA- rs121913281 C>T | | | |
|---|---|---|---|
| | | PCR cocktail for C allele | PCR cocktail for T allele |
| **Reagent** | **1x** | **10 x** | **10 x** |
| PCR master mix | 5ul | 50 ul | 50 ul |
| Forward | 0.25 ul | 2.50 ul | 2.50 ul |
| Reverse | 0.25 ul | 2.50 ul | 2.50 ul |
| Nuclease free water | 17.50 ul | 175ul | 175ul |
| Total volume | 23ul | 230ul | 230ul |
| **Add sample** | | **2ul** | **2ul** |
| | | | |
| **Total reaction volume** | | 25ul | 25ul |

**Thermocycling conditions:**

The amplification conditions used were at 95 ºC for 10 minutes followed by 40 cycles of 94ºC for 35sec, 57 ºC for 40 sec, 72 ºC for 43 sec followed by the final extension at 72 ºC for 10 minutes.

**Gel electrophoresis:**

The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator.

**Results**

**Study Population**:

All demographic features of the subjects are depicted in table 3. This population-based case–control study was done on 60 subjects among whom 30 were clinically confirmed T2D patients and 30 were healthy controls with no history of any type of cancer.

| Table 6 | | | | |
|---|---|---|---|---|
| | **T2D patients** | | healthy cont**rol** | |
| Number of subjects | N=30 | % | N=30 | % |
| Males | 20 | 66.66 | 18 | 60 |
| Females | 10 | 33.44 | 12 | 40 |
| Age >30 | 23 | 76.66 | 20 | 66.66 |
| Age <30 | 07 | 24.44 | 10 | 33.44 |

The 30 newly diagnosed T2D patients. Of 30 consecutive T2D patients, 7 (24.44%) were below or equal to 30 years age and 23 (76.66%) were above 30 years of age. Of 30 consecutive T2D patients, 20 (66.66%) were males and 10 (33.44%) were females. The 30 healthy controls 18 (60%) were males and 12 (40%) were females. Out of 30 controls, 20 (66.66%) were below or equal to 30 years age and 10 (33.44%) were above 30 years of age.

**Genomic DNA extraction from both T2D patients and healthy controls was done Qiagen Kit .The** extracted DNA was dissolved in nuclease-free water and stored at 4° C until use. Quality and integrity of DNA were checked by NanoDrop™ (Thermo Scientific, USA) and running in 1% gel electrophoresis **Nanodrop analysis of genomic DNA extracted from T2D patients:**

**The** ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. We used NanoDrop1000 Spectrophotometer to measure the purity of DNA. All DNA samples were screened for purity by measuring optical density (OD) at 260nm ($OD_{260}$) and 280 nm ($OD_{280}$). DNA concentration (ug/ml) was calculated based on the $OD_{260}$ reading as depicted in figure 1 and 2.

**Figure 1: Nanodrop analysis of genomic DNA extracted from T2D patients**

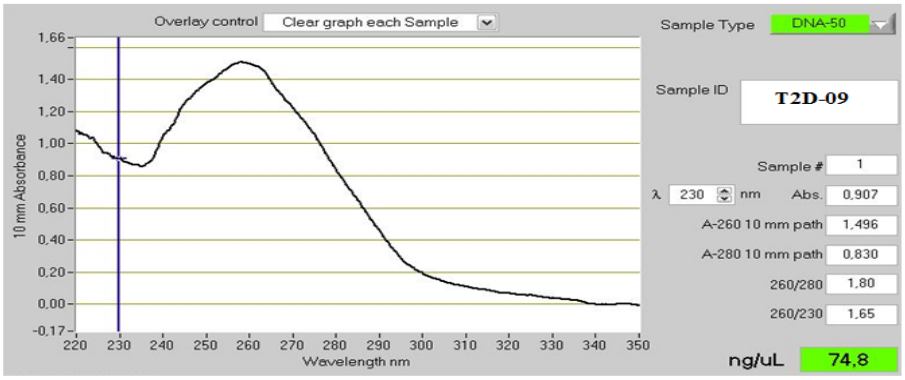
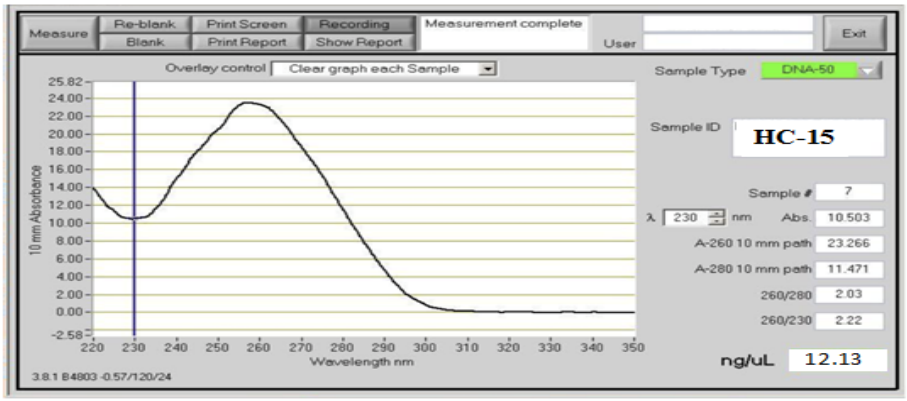
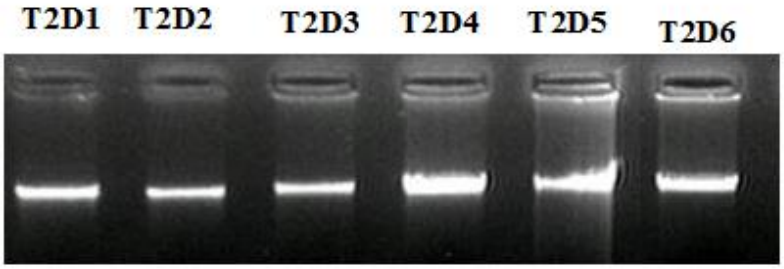



**Figure :2 : Nanodrop analysis of genomic DNA extracted from Healthy controls**




**Gel electrophoresis (1%) of genomic DNA extracted from T2D patients and healthy controls:** The quality of genomic DNA extracted from T2D patients and healthy controls was checked by running it in 1% gel electrophoresis as depicted in figure 3. It is the standard lab procedure for separating DNA by size (e.g. length in base pairs) for visualization and purification. The gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. Thus, you can accurately determine the length of a DNA segment by running it on an agarose gel alongside a DNA ladder.

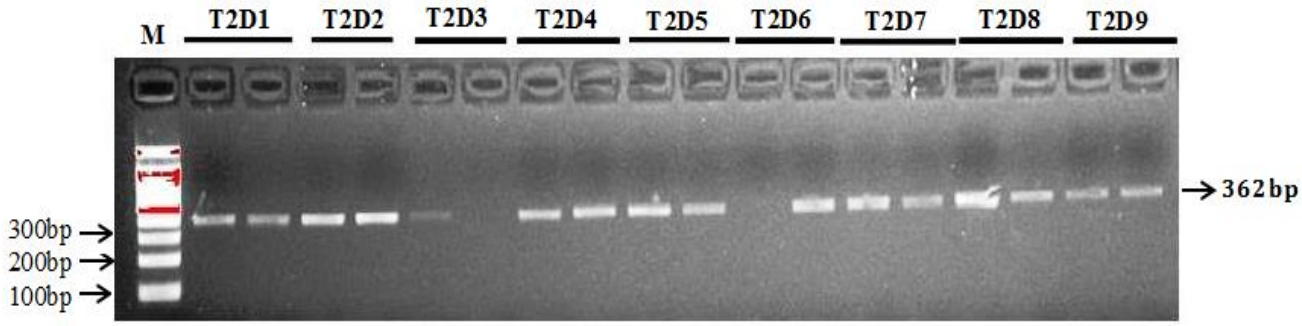
**Figure :3 Genomic DNA preparation from peripheral blood of T2D**




**Optimization of PIK3CA- rs121913281 C>T gene variation:**

The PIK3CA- rs121913281 C>T genotyping was detected by using allele specific PCR system. Gradient PCR was performed and the best temperature was determined to be at 58°C in the temperature range of 50°C to 60°C tested with a gradient PCR thermocycler. The number of cycles was increased from 30 to 45 cycles, significantly enhancing the yields of all three PCR products. Together, these changes resulted in a more robust amplification of the mutant allele and a less competing reaction from the control, as shown by the relative intensities of the corresponding bands on agarose gel electrophoresis. The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Primers F1 and R flank the exon of the PIK3CA-rs121913281 C>T gene and amplify a wild-type allele (C allele), generating a band of 362 bp and primers F2 and R amplify a mutant type allele (T allele) generate a band of 362 bp as depicted in figure 4.

**Figure :4 Detection of PIK3CA- rs121913281 C>T gene polymorphism by Allele Specific PCR in T2D and in healthy controls**




**LEGEND**

**2% gel electrophoresis**

M-100 bp DNA ladder

Homozygous CC: T2D3

Homozygous TT: T2D6

Heterozygous CT: T2D1, T2D2 ,T2D4, T2D5, T2D7,T2D8 and T2D9

Allele-specific (AS) PCR amplification has been used in combination with gel based detection to genotype-specific polymorphisms. We find it a cost-effective and reliable method that can accurately be used to rapid detection of point mutations as well as SNPs.

**DISCUSSION:**

Diabetes Mellitus (DM) is one of the major health concerns worldwide, and currently becoming too epidemic in certain countries [21]. T2D It is mainly due to interactions of many factors such as life-style as physical inactivity exercise, poor diet, overweight and genetic factors[21] . Saudi Arabia is among the top ten countries of the world with highest prevalence of diabetes mellitus. It is ranked number seven[21]. Diabetes should be treated under a close

collaboration between patients and healthcare providers in order to prevent long-term effects such as diabetic retinopathy, nephropathy, neuropathy and cardiovascular diseases[21]. Promotion and development of public awareness continued screening and early diagnosis and intervention are pivotal to boosting a positive health feedback[21]. Special care should be provided to the role of public awareness programs, community campaigns and different health educational programs in decreasing health problems caused by DM[21]. After time these efforts will assist in decrease the burden of DM[21]. It has been reported that life style interventions such as reduction of weight, diet modification, more physical activity would prevent or delay the onset of T2D in individuals at any level of genetic susceptibility [22]. PIK3CA is a gene that encodes parts of the PI3K enzyme. This enzyme constitutes an important intermediate in a signaling pathway (PI3K/AKT) involved in many important physiological processes including cell death and survival, migration, proliferation, synthesis of protein and glucose metabolism[23]. PI3K is stimulated by insulin; G-protein coupled receptor (GPCR), or cytokine receptor to produce phosphatidylinositol-3, 4, 5-triphosphate (PIP3) from the phosphatidylinositol-4, 5-bisphosphate (PIP2)[12]. The PIP3 is a an important molecule of different cellular processes including the insulin signaling pathway, apoptosis, cell cycle and others[12]. PI3K is a dimeric enzyme consists of a catalytic subunit (p110) and a regulatory subunit (p85)[13]. Then the PI3K produces a molecule that activates another enzyme called AKT. The activated AKT acts on other proteins that regulate cell processes that enhance cell growth and survival [24]. Mutations in PIK3CA lead to enhanced activation of its signaling function, thereby driving the tumorigenic process [10]. Defects in this gene have also been associated with ovarian cancer, endometrial cancer [25]. PCR is a rapid, sensitive, reliable, accurate method for amplification of DNA. It has been successfully used for diagnosis for viral[26] and bacterial infections[27]. Scientists in the past have used techniques like Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Single Strand Conformation Polymorphism (SSCP), direct DNA sequencing and AS-PCR for genotyping of specific polymorphic nucleotide loci[28]. Among these the AS-PCR could be a useful tool for genotyping, since SSCP may not be repeatable some times, direct DNA sequencing is a cumbersome, time consuming, technically demanding and costly procedure and the possibility of getting a restriction site for an enzyme could be rare for genotyping by RFLP. For genetic analysis, fast and economical assays that can be performed with standard PCR instruments are highly desirable. Compared to other genotyping techniques, such as typical PCR-RFLP analysis, real time PCR assay and DNA sequencing, the AS-PCR proved to be rapid, reliable, simple and economical assay for SNP genotyping[29-31]. AS-PCR for PIK3CA-rs121913281 C>T gene variation can used routinely as it being rapid, reliable, simple and economical assay.

## CONCLUSION:

Allele Specific-PCR for PIK3CA- rs121913281 C>T gene variation was successfully optimized. The AS-PCR technique can be used as a potential molecular tool for the detection of PIK3C gene variations in T2Ds. The assay proved to be rapid, reliable, simple and economical that does not entail any special equipment other than a thermocycler and a gel documentation system.

**Competing interests:-**

The authors declare that they have no competing interests.

**Authors' contributions:-**

All authors read and approved the final manuscript. Thanks to all authors for their support and help in this study.

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