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# Molecular Investigation of Genetic Polymorphisms in Type 2 Diabetic Patients Using Random Amplified Polymorphic DNA (RAPD-PCR)

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## Abstract :

To find DNA markers associated with Type 2 diabetes mellitus (T2D) using RAPD-PCR (Random amplified polymorphic DNA-Polymerase Chain Reaction). Peripheral blood samples were collected from 12 unrelated Iraqis with type 2 diabetes mellitus and 10 apparently healthy individuals (control subjects).

DNA was extracted and amplified using RAPD-PCR. Out of the 16 primers used, 3 did not produce amplification patterns. Seven primers produced monomorphic bands while 6 primers namely A10, A18, C5, D20, R3 and R4 produced polymorphic DNA profiles. The highest discriminatory power was produced by primers A18 and D20 reaching 25%. Primer D20 produced the highest number of bands (16) and largest molecular weight band 2.470Kb whilst primer A10 produced the lowest number of bands (3) and primer R4 the smallest molecular weight band of 0.296Kb.

Furthermore a band of 1.056 Kb was produced by primer R4 with frequency of 100% in diabetic patients and total absence in control subjects. The total length of the genome screened was approximately 118.661Kb, 40% of which represent polymorphisms.

In conclusion Polymorphisms between diabetic patients and control subjects can be detected by RAPD-PCR and DNA markers associated with type 2 diabetes mellitus can be found using the same technique.

***Key words:*** *Type 2 diabetes mellitus, RAPD-PCR, DNA markers*

## Introduction:

Type 2 diabetes mellitus is a heterogeneous clinical entity characterized by the presence of chronic hyperglycemia as a consequence of deteriorated tissue response to the biological effects of insulin (insulin resistance)(1).

It is a classical example of multifactorial disorder: The etiology of type 2 diabetes is a combination of both genetic and environmental factors (2) At least one in 10 people alive today is prone to develop diabetes at some point during their lifetimes. Most diabetic people have type 2 diabetes (T2D) and are expected to double in a generation, from 150 million in 2000 to 300 million by 2025 (3).

In Iraq the occurrence of T2D is reaching epidemic proportions with 2 million estimated people 7.43% of the overall Iraqi population (4).

Random amplified polymorphic DNA (RAPD) is a PCR based technique in which random DNA fragments are amplified by single short arbitrary oligonucleotide sequences (5). It is a rapid and applicable to any organism without prior information of the nucleotide sequence presenting only qualitative results (6). RAPD analysis can detect rearrangements, additions or deletions of DNA and ploidy changes in cells by visualizing banding shifts, missing bands or the appearance of new bands in a DNA gel electrophoresis (7).

RAPD-PCR was shown to be a sensitive technique for the genetic characterization of populations, evolutionary studies, and genetic relatedness (8). Also, RAPD was utilized in the detection of genetic alterations and instabilities in human tumors (9), (10).

The aim of the present study is to find RAPD DNA markers associated with T2D which may facilitate early diagnosis of the disease and help in providing the adequate health management offering such patients and their families valuable prognostic, diagnostic and therapeutic ben-

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efits, in addition to other studies, this one may provide an introduction to the study of T2D on molecular basis in Iraq.

# Materials and Methods:

**Subjects and controls:**

Peripheral blood was obtained from 12 unrelated Iraqis with type 2 diabetes mellitus. The mean age was 56 years.

The non diabetic control group consisted of 10 unrelated Iraqis with mean age 52.3 years (Table 1). T2D was diagnosed according to the WHO criteria (1985).

*Table 1: Characteristics of diabetic patients and non-diabetics*

| | Diabetic patients (n=12) | Control subjects (n=10) |
|---|---|---|
| Age mean (years) | **56** | **52.3** |
| Percentage female | **75%** | **30%** |
| Percentage with family history of the disease | **25%** | **30%** |
| BMI index | **26.84** | **28.33** |
| Glycated hemoglobin | **9.4** | **5.4** |

**Anthropometric measurements:**

Standing height and weight measurements were completed with the subjects wearing lightweight clothing and no shoes. Height was measured to the nearest cm and weight was measured to the nearest half kilogram (kg). Body mass index (BMI) was calculated as body weight in kilograms divided by the squared value of body height in meters (kg/m2). Informed consent was obtained from all participants.

**DNA Extraction:**

Peripheral blood was drawn from T2D patients and control subjects in acid-citrate (ACD) anticoagulant tubes and kept in -4ºC until used. DNA was extracted from whole blood using the standard phenol: chloroform method described in Sambrook et al. (1989) (11). Quantity and quality of all DNA samples were verified by UV visible spectrophotometer and electrophoresis respectively prior to RAPD- PCR.

**RAPD-PCR:**

Sixteen random primers synthesized by Alpha DNA were selected for the RAPD amplifications, primer sequences matched corresponding primers from Operon Technologies Incorporation, DNA amplifications were carried out in 25 µl reaction mixture containing 12.5 µl of 2X Green Master Mix containing 400µM each of dATP, dCTP, dGTP, dTTP and 3mM of MgCl2 (Promega), 10pmol primer and 100ng of genomic DNA.

DNA amplification reactions were performed in Eppendorf thermal cycler programmed 45 cycles of. PCR profile consisted of initial denaturation on 94ºC for 5 min and 45 cycles of 94ºC for 1 min, 36ºC for 1 min, 72ºC for 2 min and a final extension step at 72ºC for 10 min.

RAPD-PCR products were electrophoresed in 1.2% agarose gel in the presence of 1Kb DNA ladder (Promega) and were visualized by staining with ethidium bromide. Gel images were stored on computer using a gel documentation system and molecular weight estimations were carried out through Photo-CaptMwt version 10.01 computer soft ware.

**RAPD Analysis:**

An easy follow format was prepared and used for the analysis of each of the PCR products of the primers producing amplification patterns.

First, DNA bands were scored in tables for their presence 1 or absence 0 in the RAPD profiles. Primer efficiency was calculated as the number of uncommon (polymorphic) bands for each primer/total number of amplified fragments (12).

The discriminatory power percent was calculated as the number of uncommon bands for each primer/ the number of uncommon bands of all primers (13).

Band Frequency was estimated for amplification profiles produced by polymorphic primers only and calculated by counting the no. of samples sharing a specific band within each group and between the two groups. The size of the genome screened was calculated by the addition of the sizes of all the individual loci amplified by the random primers (14).

# Results:

Twenty two different individuals (12 T2D and 10 control subjects) were screened by RAPD-PCR in the search for diagnostic markers for type 2 diabetes mellitus. Of the 16 primers used, 3 did not produce amplification pattern. Seven primers produced monomorphic bands (Figure 1) while 6 primers namely A10, 18, C5, D20, R3 and R4 provided polymorphic DNA profiles, table 2 lists the primers used in this research, their sequences, GC% content and the amplification product.

Efficiency and discriminatory power of the aforementioned polymorphic primers ranged from 0.03 to 0.05 and 2.7 to 25% respectively (Table 3). Primer D20 produced the highest number of bands (16) and largest molecular

weight band (2.470Kb) while primer A10 produced the lowest number of bands (3) primer R4 produced the smallest molecular weight band of 0.296Kb. In general, a total of 107 loci were amplified by 13 primers with an average of 8 bands per primer, the total length of the genome screened was approximately 118.661Kb. Of the 107 loci, 36 were polymorphic, their size was 46.964Kb.

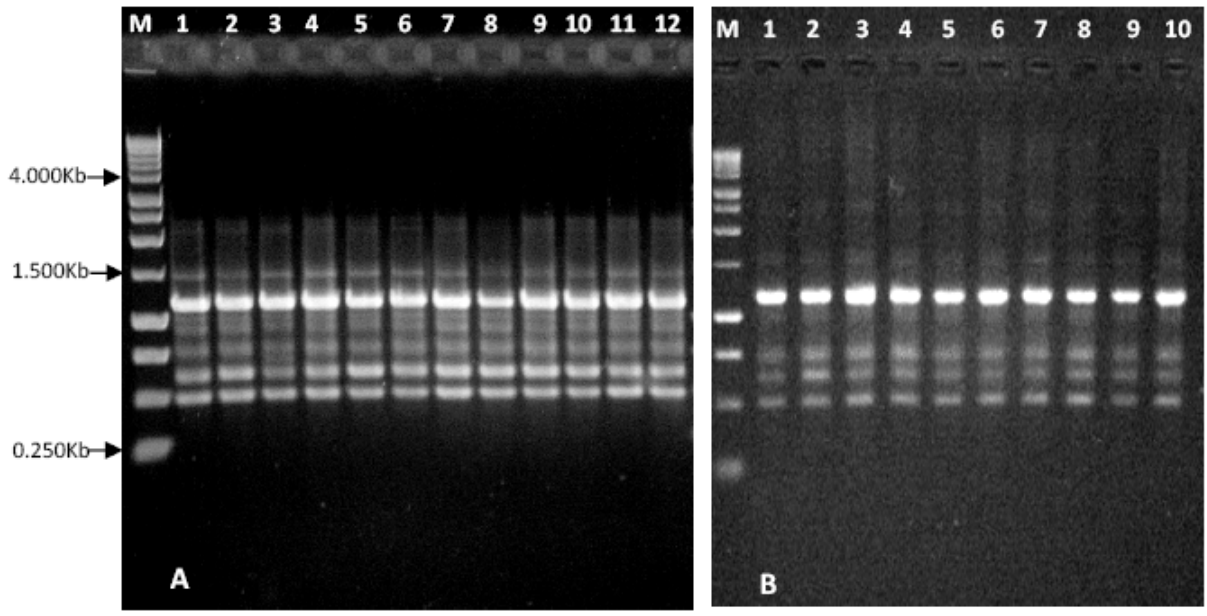


*Figure 1: RAPD amplification products generated by primer F13 resolved by electrophoresis in 1.2% agarose gel, M is 1Kb DNA ladder, A: lanes 1-12 DNA samples from diabetic patients, B: lanes 1-10 DNA samples from control subjects*

*Table (2): Primers used for the RAPD analysis, their sequences, GC content and amplification products.*

| Primer | No, of amplified bands | No, of polymorphic bands | Efficiency | Discriminatory power % |
|---|---|---|---|---|
| A10 | 3 | 1 | 0.017 | 2.7 |
| A18 | 11 | 9 | 0.05 | 25 |
| C5 | 8 | 4 | 0.03 | 11.1 |
| D20 | 16 | 9 | 0.043 | 25 |
| R3 | 10 | 7 | 0.0395 | 19.4 |
| R4 | 10 | 6 | 0.032 | 16.6 |

*Table (3): Polymorphic primers, efficiency and discriminatory power.*

| Primer | Sequence 5´-3´ | GC content% | Amplification pattern |
|---|---|---|---|
| A10 | GTGATCGCAG | 60 | Polymorphic |
| A13 | CAGCACCCAC | 70 | Monomorphic |
| A18 | AGGTGACCGT | 60 | Polymorphic |
| C5 | GATGACCGCC | 70 | Polymorphic |
| C19 | GTTGCCAGCC | 70 | Monomorphic |
| D20 | ACCCGGTCAC | 70 | Polymorphic |
| E2 | GGTGCGGGAA | 70 | No amplification |
| E7 | AGATGCAGCC | 60 | Monomorphic |
| E13 | CCCGATTCGG | 70 | No amplification |
| F13 | GGCTGCAGAA | 60 | Monomorphic |
| N16 | AAGCGACCTG | 60 | Monomorphic |
| O20 | AGCTAGCGTC | 60 | Monomorphic |
| R1 | CACACCGTGT | 60 | No amplification |
| R2 | GTCCTCGTGT | 60 | Monomorphic |
| R3 | ACGGTTCCAC | 60 | Polymorphic |
| R4 | GTCTTGGGCA | 60 | Polymorphic |

Polymorphic profiles of RAPD amplifications for diabetic patients and control subjects with primer A18 are shown in figure 2 (A) and (B) in which two bands 2.425Kb and 0.829Kb appear in different frequency and intensity.

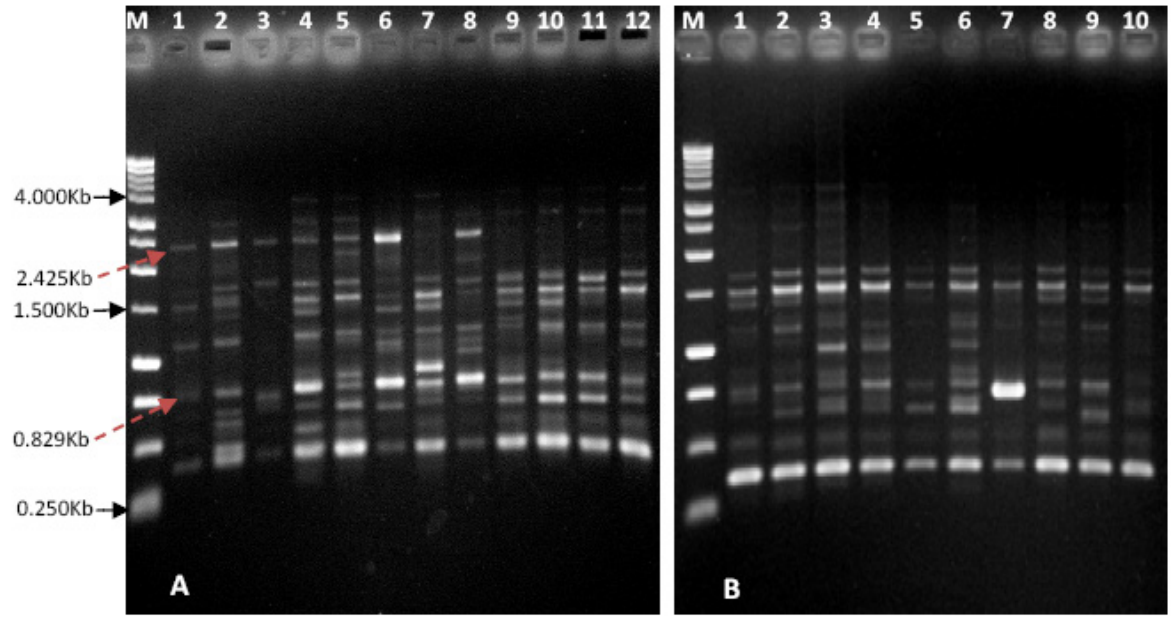


*Figure 2: RAPD amplification products generated by primer A18 resolved by electrophoresis in 1.2% agarose gel. M is 1Kb DNA ladder, A: lanes 1-12 DNA samples from diabetic patients, B: lanes 1-10 DNA samples from control subjects.*

In Figure 3, amplification pattern produced by R3, patients show a gain in 1.848Kb band namely 1,2,5,6. Both patients 2 and 6 showed loss of 1.667Kb. Patients 9, 11 and 12 showed a 1.700Kb band loss.

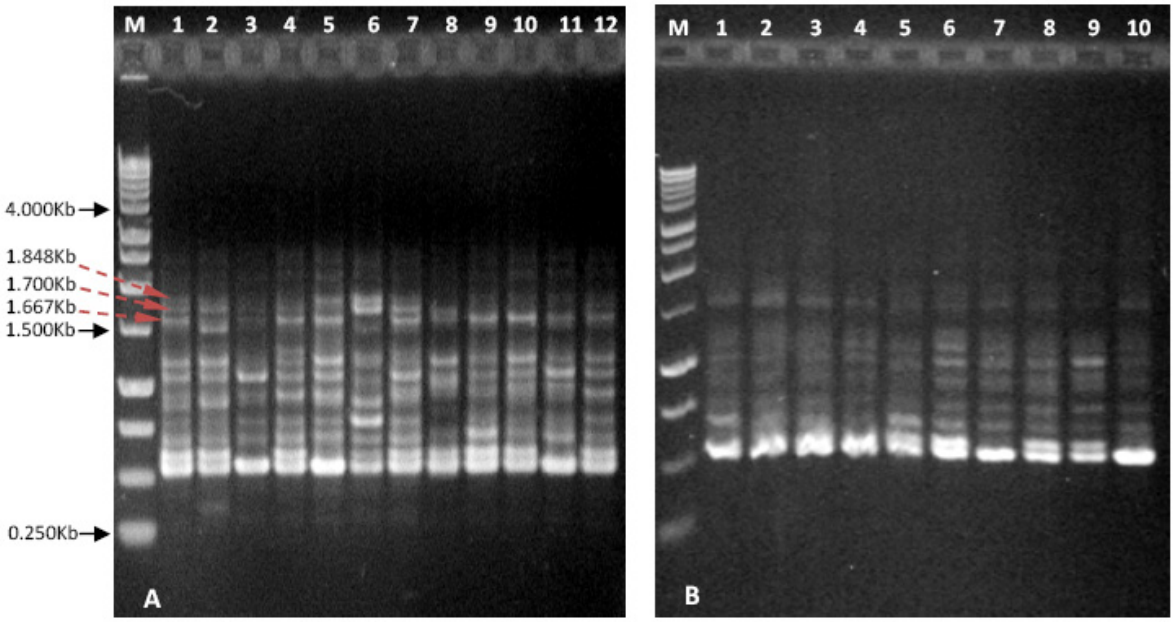


*Figure 3: RAPD amplification products generated by primer R3 resolved by electrophoresis in 1.2% agarose gel, M is 1Kb DNA ladder, lanes 1-12 DNA samples from diabetic patients, B: lanes 1-10 DNA samples from control subjects*

The polymorphic primer R4 showed significant frequency of 1.056Kb band in diabetic patients in comparison to control subjects (Figure 4). The band 1.056Kb was present in 100% in patients and was completely absent in the control group. In general polymorphic bands produced by A10, A18, C5, D20, R3 and R4 have different frequencies in diabetic patients and control subjects (Table 4).

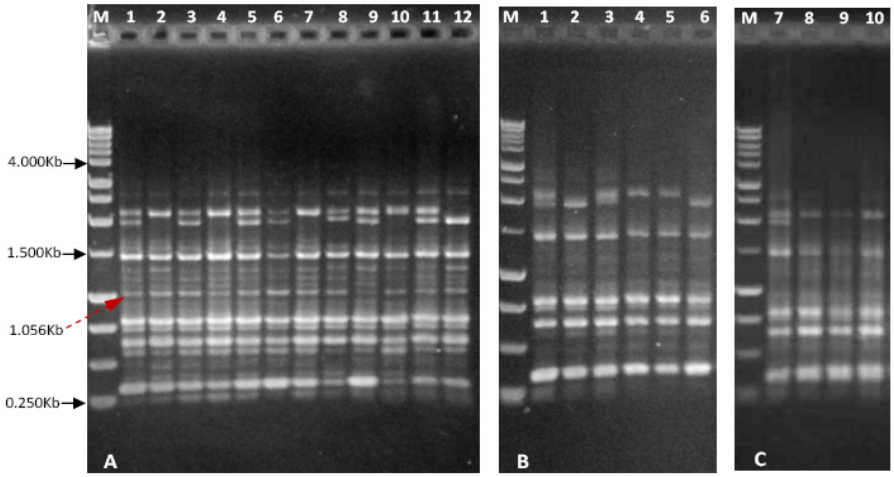


*Figure 4: RAPD amplification products generated by primer R4 resolved by electrophoresis in 1.2% agarose gel, M is 1Kb DNA ladder, lanes 1-12 DNA samples from diabetic patients: lanes 1-6 DNA samples from control subjects, C: lanes 7-10 DNA samples from control subjects*

*Table 4: Frequency of polymorphic bands in diabetics and control subjects*

| Primer | Polymorphic band size (Kb) | Frequency of polymorphic bands (%) | |
|---|---|---|---|
| | | Patients | Control subjects |
| A10 | 1.000 | 100 | 20 |
| A18 | 2.425 | 58.3 | 30 |
| | 1.800 | 91.6 | 100 |
| | 1.550 | 66.66 | 100 |
| | 1.445 | 50 | 80 |
| | 1.240 | 75 | 80 |
| | 1.044 | 25 | 40 |
| | 0.829 | 91 | 90 |
| | 0.670 | 100 | 70 |
| | 0.766 | 0 | 20 |
| C5 | 2.300 | 50 | 0 |
| | 1.967 | 75 | 0 |
| | 0.645 | 33 | 100 |
| | 0.600 | 41 | 70 |
| D20 | 2.470 | 100 | 80 |
| | 2.197 | 91.6 | 80 |
| | 2.026 | 8.3 | 0 |
| | 2000 | 16.6 | 0 |
| | 1.678 | 91.6 | 100 |
| | 1.468 | 25 | 0 |
| | 1.387 | 25 | 0 |
| | 1.125 | 22.7 | 0 |
| | 0.500 | 16.66 | 0 |
| R3 | 1.848 | 33.3 | 0 |
| | 1.700 | 66.6 | 0 |
| | 1.667 | 75 | 100 |
| | 1.105 | 91.6 | 100 |
| | 0.966 | 91.6 | 100 |
| | 0.814 | 91.6 | 100 |
| | 0.629 | 83.3 | 70 |
| R4 | 2.190 | 91.6 | 90 |
| | 2.000 | 66.66 | 50 |
| | 1.056 | 100 | 0 |
| | 0.669 | 91.66 | 100 |
| | 0.592 | 100 | 40 |
| | 0.296 | 83.3 | 100 |

## Discussion:

Polymorphisms between diabetic patients and control subjects were detected among the amplification products by 6 out of 16 primers.

Highest primer efficiency was produced by A18 followed by D20, primer efficiency values range from 0 to 1 and is defined as the measure of the primer capacity to produce polymorphisms (15). The primer ability to show polymorphisms in comparison to polymorphisms shown by all primers is known as primer discriminatory power (13) both primers mentioned above showed high discriminatory power reaching to 25% (Table 3).

Band intensity is one of the factors used in the analysis of RAPD profiles some patients and control subjects showed markedly intense bands, for example amplification patterns produced by primer A18 in figure 2 (A)(B), patients 4,6,8 show a more intense 0.829Kb band than others . Also a 2.425Kb band appear more intense in patients 2, 6 and 8 than others. In the same figure (B) a very intense 1.044Kb band appear in control subject 7.

In general patients 6, 8, 9 and 10 show frequent several strong bands in amplification patterns produced by many primers such as D20, O20 and N16 (results not shown) however these patients do not seem to share a specific clinicopathology. Technical errors in this regard are unlikely because other DNA bands in the mentioned profiles are in consistent intensity with other samples. Band intensity variations are related to copy number change and so the observed increased intensity is a result of hyperploidy (14). In the amplification products generated by primer R3 (Figure 3), 33.3 % of patients showed gain in 1.848Kb band namely 1,2,5,6. In addition both patients 2 and 6 showed loss of 1.667Kb. Patients 9, 11 and 12 showed a 1.700Kb band loss and 0.629Kb loss was seen in patients 3 and 11.

Again patients do not appear to share a specific clinicopathology. Similarities between several patients whether in band intensity, gain or/and loss of amplified fragments may reflect the fact that diabetes mellitus is a multifactorial disease with many genes affected and these similarities maybe occurring in the same genes in these patients.

Close frequencies in most of the polymorphic bands between patients and control subjects as shown in table 4 is acceptable as most of the genomic DNA is similar and majority of the amplified fragments produced by arbitrary primers are identical between individuals (16) thus monomorphic patterns may reflect amplifications in homogenous genomic regions while slight differences in the percentages of polymorphic bands maybe interpreted as heterozygous regions of amplification (Figure 4).

Information about particular DNA sequences or functional gene loci are not provided by RAPD-PCR observations however genetic polymorphisms seen maybe suggestive of loci that contribute to diabetes. Variations at an undetermined number of genomic sites in addition to environmental factors influence individual susceptibility in most people with T2D. The list of susceptible genes involved in the pathogenesis of diabetes is large including but not limited sets of genes with proteins implicated in beta-cell, adipocyte and/or hepatocyte development and function, in insulin-signaling or hypothalamic regulation (17). Observed variations and polymorphisms between diabetic patients and control subjects specially those present in higher percentages may be occurring on such susceptibility genes as band shifting is due to different types of mutations including rearrangements, additions or deletions that occur in the DNA region of amplification in sequences flanked by the priming sites or with in the priming site itself, such variations may also result in the appearance of new bands or disappearance of preexisting ones (5). Bands such as 1.000, 1.967, 1.700, 0.592Kb produced by primers A10, C5, R3 and R4 respectively fall into this category i.e. are examples of bands that appear in highly different frequencies in patients in comparison to control subject group and may prove helpful as diagnostic markers in addition to other well identified markers if more studies are conducted to detect their sites and on which genes they are present in the human genome. Whilst the band 1.056Kb produced by primer R4 serves best the objective of this study and may be considered as a diagnostic DNA marker in patients with T2D in view of the fact that it appeared in 100% of patients and was completely absent in control subjects. However in both cases more research is needed to support these results and further identification of DNA fragments need to be conducted to see if they are of relevance, probably a sample with a larger size will offer a better study design, cloning and sequencing of the loci amplified in this study is one way that might reveal mutations of functional importance to the disease and may provide a confirmative conclusion.

Genetic information plays a role in the clinical care of monogenic and syndromic forms of diabetes however molecular diagnosis of multifactorial T2D is still premature because of the limited prognostic value of variants involved in the susceptibility to this disease (18).

The sensitivity and reliability of RAPD-PCR is validated by the fact that polymorphisms screened cover a wide genome range including both susceptible regions and or unrelated regions to diabetes. In conclusion RAPD-PCR showed qualitative differences in diabetic patients in comparison to non-diabetics.

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## References:


1. Saltiel A.R. and Khan C.R. (2001). Insulin Signaling and the regulation of glucose and metabolism. Nature 414: 799-806.
2. Toye A. and Gauguier D. (2003). Genetic and functional genomics of type 2 diabetes mellitus. Genome Biology 4:24.
3. Zimmet P., Alberti K.G.M.M. and Shaw J. (2001). Global and societal implications of the diabetes epidemic. Nature 414: 782–787.
4. Mansour A.A., Wanoose H.L., Hani I, Abed-Alzahrea A., Wanoose H.L. (2007). Diabetes screening in Basrah, Iraq: A population-based Cross sectional study. Diabetes Res Clin Pract 79: 147-50.
5. Williams J. G., Kubelik A.R., Livak K.J., Rafalsky J.A. and Tingey S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Res. 18:6531-6535.
6. Ali A.B. (2003).Detection of DNA alteration in abnormal phenotype of broiler chicken male by random amplified polymorphic DNA (RAPD). African journal of Biotechnology vol2 (6):153-156.
7. Ong T.M., Song B., Qian H.W., Wu Z.L. and Whong W.Z. (1998). Detection of genomic instability in lung cancer tissues by random amplified polymorphic DNA analysis. Carcinogenesis19:233-235.
8. Al-Bustan S.A., Al-Naqeeb M.A., Murad N.Y. and Al-Alawi A.F. (2001). Genetic variation of inbred laboratory rats by RAPD-PCR. Kuwait J. Sci. Eng. 28 (2).
9. Arribas R., Capella G., Tortola S., Masramon L., Grizzle W.E., Perucho M. and Peinado M.A. (1997). Assessment of genomic damage in colorectal cancer by DNA fingerprinting: prognostic applications. J.Clin.Oncol. 15: 3230-3240.
10. Paditaporn R., Riengrojpitak S., Punyarit P., Chindavijak S., Karalak A. and Petmitr S. (2007). Genetic instability in cervical cancer detected by arbitrary primed polymerase chain reaction. Asian Pacific Journal of Cancer Prevention vol 8:109-112.
11. Sambrook J., Fritsch E.F. and Maniatis T. (1989). Molecular Cloning: A Laboratory Manual. 2nd edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
12. Hunter R.P. and Gaston M.A. (1988). Numerical index of discriminatory ability of typing systems: an application of Simpsons's index of diversity. J.clin. Microbiol. 26: 2456-2466.
13. Grundman H., Scheider C., Hartung D., Daschner F.D. and Pitt T.L. (1995). Discriminatory power of three based typing techniques or P. aeruoginosa. J.clin. Microbiol 33(3): 528-534.
14. Singh K.P. and Roy D. (2004). Somatic mutations in stilbene estrogen-induced Syrian hamster kidney tumors identified by DNA fingerprinting. Journal of carcinogenesis 3:4.
15. Newton C.R. and Graham A. (1997). Polymerase Chain Reaction. Bios scientific publishers. Oxford, UK.
16. Theodorakism C.M., Bickman J.M., Elbi T., Shugart L.R. and Chesser R.K. (1998). Genetics of radionuclide -contaminated mosquito fish populations and homology between Gambusia affinis and G. holbbrooki. Environ. Toxicol. Chem. 17:1992-1998.
17. McCarthy M.A. (2004). Progress in defining the molecular basis of type 2 diabetes mellitus through susceptibility-gene identification. Human molecular genetics Vol.13, Issue 1:33-41.
18. Haga S.B., Khoury, M.J. and Burke, W. (2003). Genomic profiling to promote a healthy lifestyle: not ready for prime time. Nat. Genet., 34,347–350.


# التحري الجزيئي عن التغايرات الوراثيه في مرضى السكري نوع 2 بأستخدام تضاعف الدنا متعدد الاشكال العشوائي لانزيم بلمرة الحامض النووي


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**الخلاصة:**

تهدف هذه الدراسه الى ايجاد مؤشرات وراثيه من الحامض النووي ذات علاقه بداء السكري من النوع الثاني بأستخدام تضاعف الحامض النووي متعدد الاشكال العشو ائي لانزيم بلمرة الحامض النووي. جمعت نماذج من الدم المحيطي لاثني عشر مريضا بداء السكري النوع الثاني لاتربط بينهم علاقه قربى و عشرة اشخاص اصحاء بالنسبه للمرض كنماذج سيطره بعد استخلاص الحامض النووي وتكثيره بتفاعل البلمره.

من بين الـ 16 بادئا التي أستخدمت في هذه الدراسه, ثلاثه بادئات لم تعطي اي نتيجه تضاعف, فيمااعطت سبعة بادئات نمط تضاعفي غيرمتغايرفي حين اعطت ستة بادئات نمط تضاعفي متعدد الاشكال وهي 10A, 18A, 5C, 20D, 3R و 4R. سجلت اعلى قوه تمييزية بين البادئات للبادئ 18A و 20D حيث وصلت الى 25% و اعطى البادئ 20D اكبر عدد من الحزم (16حزمه) كذلك اعطى اكبر حجم بين الحزم المتضاعفه 2.470 كيلو قاعده بينما اعطى البادئ 10A اقل عدد من الحزم و سجلت للبادئ 4R اصغر حزمه متضاعفه بحجم 0.296 كيلو قاعده.

اضافة الى ذلك انتجت حزمه من للبادئ 4R بتكرار 100% في مرضى السكري و غابت تماما في نماذج السيطره .تم التحري عن ما يقرب 118.661 كيلو قاعده من الحامض النووي الكلي للانسان مثلت 40% منها تغايرات وراثيه.نستنتج بانه يمكن التحري عن التغايرات الوراثيه في مرضى السكري نوع 2 باستخدام طريقه تضاعف الحامض النووي متعدد الاشكال العشوائي لانزيم بلمرة الحامض النووي كما وانه بالامكان ايجاد مؤشرات وراثية من الحامض النووي لمرضى السكري نوع 2 بأستخدام نفس التقنيه.