Archives of Advances in Biosciences 2021:12(3) doi.org/10.22037/aab.v12i3.34675

Original Article

# Molecular Investigation of c-MYC Oncogene Amplification in Iranian Diabetics

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**Article Information**
Received:2021-04-27
Revised: 2021-06-01
Accepted: 2021-06-11

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**Cite this article as**:

Jalal Abadi A, Bidoki S K, Ghoraeian P, Gholami M A, Hashemi F . Molecular Investigation of c-MYC Oncogene Amplification in Iranian Diabetics..Archives of Advances in Biosciences 2021:12(3)

## Abstract

**Introduction:** There is growing evidence revealing that genetic factors could be involved in the etiology of insulin resistance and diabetes. Recent studies now suggest that c-MYC oncogene amplification in beta-cells can cause downregulation of insulin gene expression and leading to diabetes. The present study was carried out to examine gene amplification level of c-MYC gene in healthy individuals compared to those with diabetes.

**Materials and Methods** : This case control study consisted of 70 subjects (34 diabetic patients and 36 healthy controls). The genomic DNA was extracted from blood samples using standard phenol-chloroform method, and Differential Quantitative PCR (DQ PCR) was accomplished. Subsequently, the mean Band Intensity Ratio (BIR) of c-MYC to γ-IFN was applied to determine the amplified products' molecular band intensity. The Real-time PCR was used to reconfirm the obtained results.

**Results**: The mean BIR of c-MYC to γ-IFN was 1.29 in diabetics, whereas in healthy individuals, the mean BIR was equal to 1.16.The findings indicate that the mean BIR in diabetics was almost 1.1 times higher than healthy controls; however, it was not statically different (P-value= .168).

**Conclusions**: Since no significant difference was found in terms of BIR of c-MYC to γ-IFN in case and control groups, it can be concluded that c-MYC may not have a role to trigger the disease in this limited Iranian population. Further studies and larger statistical populations are needed to confirm our findings. This study showed that the DQ PCR technique could be reliable to screen gene amplification in large populations.

**Keywords:** Diabetes; c-MYC oncogene; Gene amplification

## 1. Introduction

Diabetes Mellitus (DM), known as diabetes, is a group of metabolic disorders impairing the ability of the body to process blood sugar. Diabetes occurs because not enough insulin by the pancreas is produced or there is inadequate response to the insulin

produced [1]. Some types of diabetes, like type I and type II, are more prevalent than others [2].

In type I diabetes, the pancreas cannot produce enough insulin due to β-cells loss. As a result, individuals with type I diabetes are insulin-dependent; in fact, they must take artificial insulin daily to break down the carbohydrates [3].

In contrast, type II diabetes begins with insulin resistance that affects the way body takes insulin. While body still produces insulin, unlike type I, insulin receptors no longer respond to the insulin appropriately [4].

As of 2017, the most common form of diabetes in the general population was type II diabetes; however, a significant minority had type I diabetes [5]. According to the results published by the International Diabetes Federation (IDF), approximately 425 million people have diabetes in the world, with type II diabetes making up around 90% of the cases. This represents 8.8% of the adult population, with almost equal prevalence in both men and women [6].

Although diabetes is not yet curable, it can be successfully controlled when detected early [7]. Thus, early diagnosis and effective treatment of the disease can reduce its severe complications and death. Recent studies and analyses substantiate enough evidence that genetic factors play a decisive role in incidence and development of diabetes [1]. As a result, identifying the candidate genes and genetic variation involved in the incidence of diabetes is of crucial importance [8].

The growing interest in identifying genes and molecular processes associated with the appearance of diabetes has led to finding 28 candidate genes so far [9]. Lately, working on mice models has revealed that the c-MYC oncogene is one of the genes implicated in the loss and dysfunction of insulin-producing beta-cells in diabetes. It is intriguing that such findings increase the probability that c-MYC might be a pivotal contributor to diabetes through disrupting the process of insulin production due to hyperglycemia [10].

However, given the fact that diabetes is a heterogeneous disease, it is difficult to attribute a specific pathogenic role to c-MYC or any other given single factor. Meanwhile, c-MYC is an attractive target gene potentially in diabetes and complex diseases [11].

Unlike other studies conducted on mice tissue, the current study aimed to examine the association between the gene amplification level of the c-MYC and the risk of diabetes on the peripheral blood of human samples, using DQ PCR and Real-time PCR approaches

## 2. Materials and Methods

### Study population

The case group consisted of 34 patients (13 males and 21 females), eight with type I, and 26 with type II diabetes. The participants were recruited among the patients referring to Imam Khomeini Hospital in Karaj. The control participants of 36 people with no evidence of disease were chosen from blood donors referring to Dr. Bidoki Genetic Center in Karaj.

### DNA Extraction

Total DNA was isolated from .4 ml of EDTA treated blood samples, pretreated with proteinase K using standard protocol for extraction with phenol-chloroform. Thereafter, the quantity and quality of total genomic DNA were determined using UV–visible spectrophotometer (UV-1601, Shimadzu, Japan).

### Differential Quantitative PCR

Primers were designed using the NCBI Primer-BLAST tool (Table 1) [12]. The reaction mixture for Differential Quantitative PCR consisted of 1µl of extracted DNA (100ng), 2.5 µl of 1 X PCR Buffer (100mM Tris-HCl (pH 9.0); 500mM KCI; 1% Triton x-l00), 1.0 µl of 25 mM MgCl2, 2.0 µl of each forward and reverse primers (c-MYC-239 bp and γ-IFN-85 bp genes) , 4.0 µl of dNTPs (1.25 mM dCTP, dATP, dTTP and dGTP), .2 µl of DNA Taq polymerase (CinnaGen Co.Ltd) (5 units/µl), and 8.3 µl four times deionized distillation water, in a final volume of 25 µl. DNA was amplified by using a Peltier thermal cycler. The PCR temperature profile was done for 40 cycles, with the first cycles of 94°C for 8 min followed by 40 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min, and the final extension period of 72°C for 10 min.

The PCR products were manifested by subjecting samples to electrophoresis using 3.5% agarose gel in 1×TAE buffer. DNA band was stained with ethidium bromide; then 5 µl of the PCR product and 3 µl of loading dye was loaded into slots. In the next stage, 3 µl of 50 bp DNA ladder (CinnaGen) was loaded into the gel to estimate the size of amplification products. The amplified products were determined and then visualized by UV Transillumination.

### Real-time PCR

Designed primers for Differential Quantitative PCR were also used in this technique (Table 1). Quantitative Real-time PCR was done by QIAGEN SYBR® Green PCR Kit according to the manufacturer's instructions in a final volume of 20ul. The reaction mixture consisted of 1µl of extracted DNA (100ng), .5µl of each forward, and reverse primers (10pmol), and 10µl of MasterMix Qiagen, and the mixture run on a Rotor-Gene Q (Qiagen).

### Statistical Analysis

Clinical and laboratory data were expressed as frequencies, mean ± S.D, or percentages. The independent samples test was used to compare quantitative data in case and control groups of DQ PCR techniques. A two-tailed $P<.05$ was considered statistically significant. DQ PCR results were analyzed by SPSS version 20.0 (SPSS, Chicago, IL, USA). Real-time PCR results were submitted to GraphPad for analysis.

## 3. Results

### Detection of c-MYC amplification by DQ-PCR

DQ-PCR was carried out on 70 participants (34 diabetics and 36 healthy controls). Among 34 patients with diabetes, the c-MYC gene amplification compared to the γ-IFN single gene as control was higher in 28 patients (82.4%). However, in 5 out of 34 patients (14.7%), the gene amplification remained constant. The results revealed that the gene amplification of c-MYC could increase in patients with either type I or type II diabetes. Findings showed that the Band Intensity Ratio (BIR) in diabetics varied between .93 and 2.34 (mean=1.29), while in healthy individuals it was reported to be between .87 and 2,27 (mean = 1.16). This indicated that the c-MYC gene amplification in diabetics was almost 1.1 times higher (Figure 1).

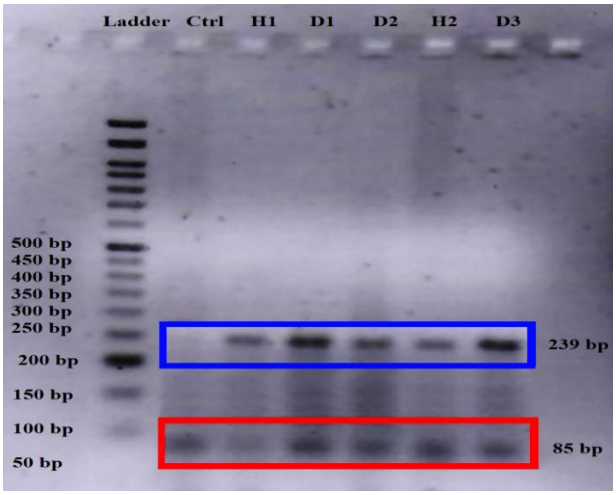


**Figure 1**. Gel electrophoresis result for the c-MYC gene amplification compared to γ-IFN. From the left, 50 bp ladder, Control, (H1-H2): healthy controls (D1-D3): diabetic samples with amplification. The box on top shows the c-MYC band, and the one at bottom shows the γ-IFN housekeeping gene band (c-MYC: 239 bp and γ-IFN: 85 bp).

In addition, the Kolmogorov–Smirnov test was performed to check the normality or non-normality of data distributions. Then, histogram diagrams of BIR were drawn to compare c-MYC oncogene amplification to y-IFN in healthy and diabetic groups. The results showed that the BIR between c-MYC and γ-IFN of Multiplex technique in both case and control groups were almost the same, although it was slightly higher in diabetic patients.

Relationship of c-MYC amplification with Age, Gender, and Type of Diabetes

Pearson correlation of non-normal distributions analysis of DQ PCR showed that the *c-MYC* gene amplification increased within each age stratum. The highest gene amplification (86.6%) was found in the third age stratum, including people aged 60 years and over. Also, the lowest gene amplification (73.3%) was observed in those with the age range between 40 and 60; however, no significant statistical difference (*P*-value=.35) was found (Table 2).

Spearman correlation of normal distributions was used to investigate the relationship of c-MYC oncogene amplification with both gender and type of diabetes. The results indicated that the *c-MYC* gene amplifications were almost the same in both males and females. The gene amplification increased by 84.6% in males and 85.0% in females, which was not significantly different. (*P*-value=.94) (Table 2).

Regarding the type of diabetes, Spearman correlation results indicate that the c-MYC gene amplification was observed in both groups with type I and type II diabetes (75.0% and 84.6%, respectively). No significant difference (*P*-value=.46) was found between them. This implies no association between the type of diabetes and the *c*-MYC gene amplification (Table 2). Finally, the Phi correlation test measured the

correlation or independence of gene dose amplification with prognostic factors.

## Confirmation of DQ PCR with Real-time PCR

Five samples were randomly selected from each patient and healthy group and were set aside for Real-time PCR to confirm the results obtained from DQ PCR. The results confirmed the previous findings, and no significant difference was found between the results obtained from these two techniques (*P*-value=.68) in diabetics compared to healthy ones (Figure 3). The results of the melting curve analysis of the Real-time PCR indicated the presence of distinct melting peaks in both the *c-MYC* and *γ-IFN* genes as target and housekeeping genes, respectively (Figures 4). These results confirmed the high specificity of primers applied in this study; the results were analyzed by GraphPad analysis software.

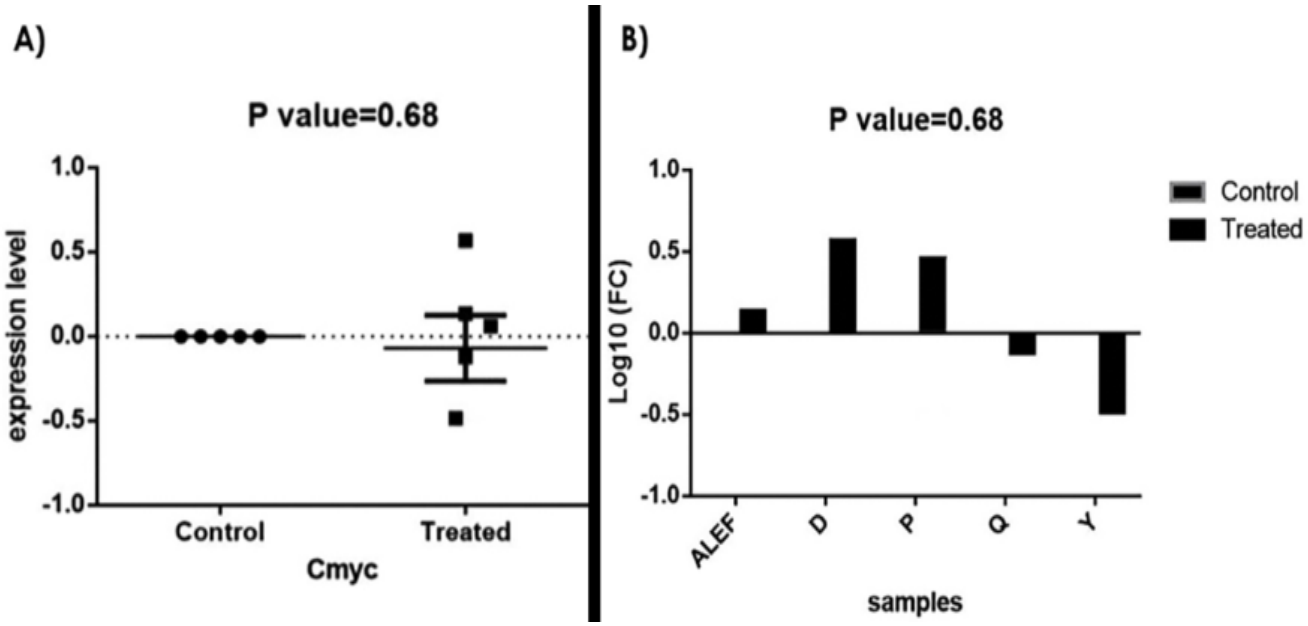


**Figure 2.** Real-time PCR diagrams results. A) Expression level in control and treated groups, as observed out of five patients, three patients have gene amplification B) Calculation of fold change of gene expression in logarithmic form in five randomly selected patients (ALEF, D, P, Q, and Y).

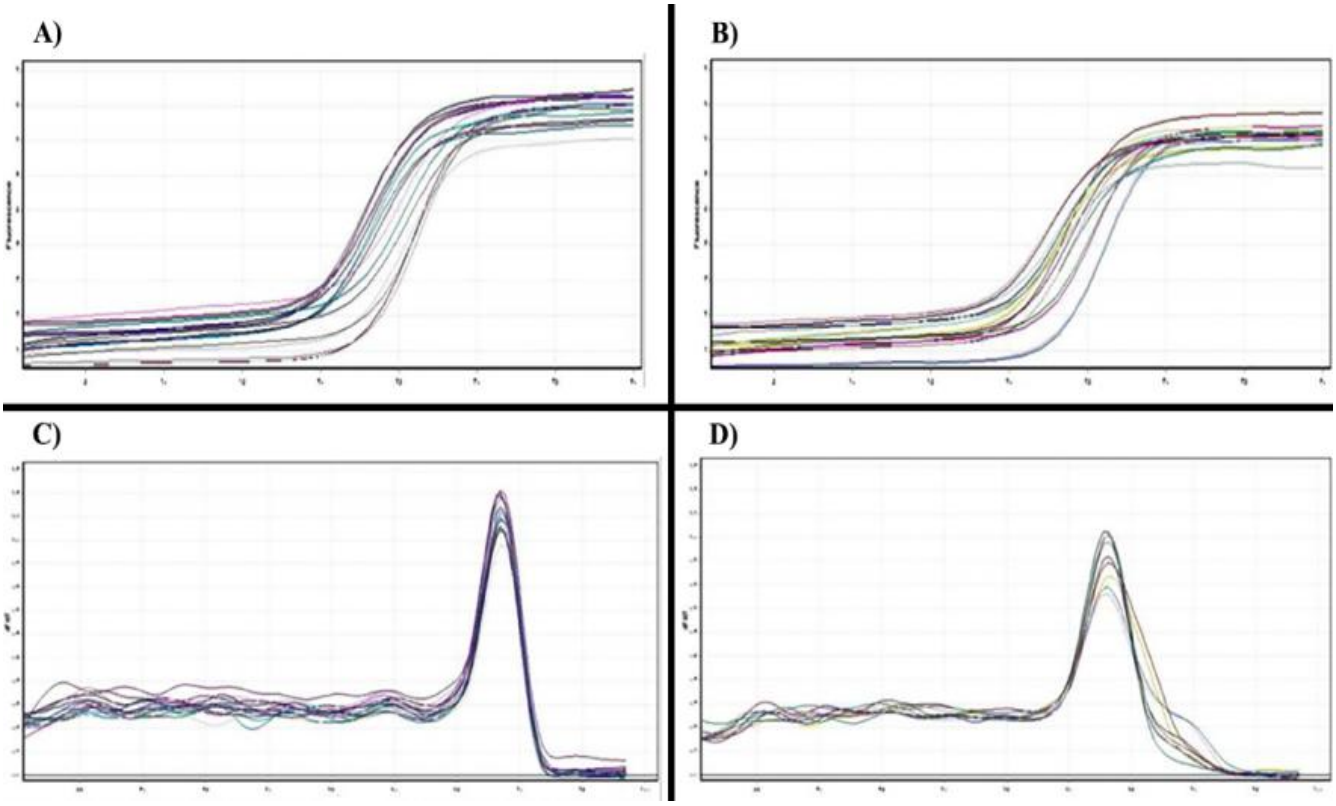


**Figure 3.** Real-time PCR Amplification plot of A) c-MYC gene and B) γ-IFN gene (as housekeeping), using the SYBR Green. Melting curve diagram of C) c-MYC gene and D) γ-IFN gene yields distinct melting peaks. In total, five randomly selected samples were chosen from both healthy and diabetic groups to verify the result of the DQ-PCR technique.

## 4. Discussion

Nearly half a billion people live with diabetes today and it is predicted that the number will rise continuously. Meanwhile, low- and middle-income countries carry almost 80% of the diabetes burden. Although public health bears heavy financial costs, hindering socio-economic development, the prevention of diabetes remains considerably underfunded. In 2017, diabetes led to about 3.2 to 5.0 million deaths, making it the eighth leading cause of death worldwide.

In order to make great strides in preventing diabetes worldwide, more comprehensive investigation is required to strengthen the evidence base and gather deeper knowledge as a basis for methods and programs handling the diabetes epidemic. Recent evidence suggests that the MYC family of cellular oncogenes gene plays a decisive role in cell cycle progression, apoptosis, and cellular transformation. The MYC proteins are regulatory proteins with a high binding affinity to E-box-related sequences

[13]. According to the findings, the c-MYC is a basic helix-loop-helix (bHLH) leucine zipper (bHLH-Zip) transcription factor widely studied as an overexpressed oncogene due to hyperglycemia, and its overexpression prevented both insulin gene transcription and glucose-stimulated insulin secretion. In gel-shift assays, c-Myc Bind to the E-box in the insulin gene promoter Bind Both c-Myc and NeuroD can be attached to the E-box element in the insulin promoter, but unlike NeuroD, the c-Myc transactivation domain could not activate insulin gene expression. Increased expression of c-Myc in beta-cells suppresses the insulin gene transcription by inhibiting NeuroD-mediated transcriptional activation. This mechanism may explain some of the beta-cell dysfunction found in diabetes[14]. This is also known as an essential factor for normal cell cycle progression. Significantly, any dysfunction in c-MYC activation pathways may inhibit differentiation and subsequently lead to cell hypertrophy [15]. The finding showing c-MYC increases are associated with beta-cells hypertrophy suggests that c-MYC plays a vital role in regulating blood glucose; however, other signaling pathways or other chemicals, like hormones, need to be considered as well [14],[16].

Studies from animal models suggest that pancreatic beta-cells failure is a basic principle to the pathogenesis of all forms of diabetes. In animal models of diabetes, it has been shown that c-MYC amplification is significantly lower in normal transgenic mice. Additionally, the c-MYC amplification is increased in diabetic mice [17].

A study on MYCER transgenic mice's liver tissue with increased c-MYC expression showed that c-MYC plays a crucial role in the beta-cell failure. Also, c-MYC activity in beta-cell in MYCER mice can cause beta-cell apoptosis and diabetes. However, a key issue is whether hyperglycemia plays a role in the induction of beta-cell apoptosis or whether beta-cell death occurs alone due to c-MYC activity [18].

Another study examined the expression of the c-MYC oncogene in the induction of oral cancer in diabetic and normal type I Sprague Dawley rats. The c-MYC oncogene was higher in diabetics although the expression pattern was the same. In contrast, c-MYC expression was significantly higher in diabetics. Diabetes appears to enhance c-MYC presentation only in the early stages of oral cancer [19].

Blood glucose was examined in transgenic RIP-II / MYC mice. Their blood glucose showed that overexpression of c-MYC in transgenic mice compared to wild ones is associated with changes in beta-cell proliferation and the formation of islets of Langerhans, and decreased expression of the insulin gene. The blood sugar of transgenic mice was 36% higher than their wild counterparts. The results indicated that after the overexpression of c-MYC in mice, diabetes increased significantly [10].

Our study tested the hypothesis proposing the c-MYC gene plays an essential role in the incidence of diabetes in humans by affecting the function of beta-cell. To do so, the c-MYC gene amplification was assessed against the y-IFN housekeeping gene in the control and diabetic groups by Differential Quantitative PCR. Further assessments were carried out to compare healthy and diabetic

individuals (5 samples were selected randomly) by Real-time PCR. Both techniques showed the DNA level increased slightly in diabetics when compared to healthy individuals. Despite finding valuable results, P-values were .168 and .68, respectively. Therefore, discordant from the previous research which confirmed the relationship between c-MYC oncogene overexpression and diabetes, we did not observe any significant relationship between diabetes and c-MYC oncogene amplification. In other words, our results did not verify this relationship in this designated population. Further studies are required to scrutinize this relationship in other populations.

## 5.Conclusion

The results revealed that the c-Myc oncogene amplification was slightly higher in diabetics compared to the control group. As the case group consisted of individuals with type I and type II diabetes, it can be concluded that the results are generalizable to both types of diabetes. However, the gene amplification was not significantly different in the case group in comparison with healthy group (*P*-value= .168 and .68), implying that we can use effective techniques such as DQ PCR to screen for gene amplification in developing countries.

## Conflict of interest:

The authors declare that there is no conflict of interest.

## Acknowledgment

We thank Gene Afzar Medical Genetics Center, Dr. Bidoki Medical Genetics Center, and Imam Khomeini Hospital for their support and cooperation.

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| Primer | Sequence (5/-3/) | Tm (o C) | Position |
|---|---|---|---|
| c-MYC F | GCACTGGAACTTACAACACC | 61.1 | 1025-1263 |
| c-MYC R | TAGGCATTCGACTCATCTCA | 60.5 | |
| γ-IFN F | AGTGATGGCTGAACTGTCGC | 62 | 4302-4386 |
| γ-IFN F | CTGGGATGCTCTTCGACCTC | 63.8 | |

**Table 1.** The primers' sequence and melting temperature (Tm)

| Specification | Variable | Frequency (%) |
|---|---|---|
| Number of patients | Person | 34 person |
| Age | 40≤ | 4 persons (12%) |
| | 40-60 | 15 persons (44%) |
| | ≥60 | 15 persons (44%) |
| Sex | Man | 21 persons (62%) |
| | Woman | 13 persons (38%) |
| HbA1C | 7≤ | 5 persons (15%) |
| | 7-10 | 17 persons (50%) |
| | ≥10 | 12 persons (35.3%) |
| FBS | 150≤ | 13 persons (38.2%) |
| | 150-300 | 13 persons (38.2%) |
| | 300≥ | 8 persons (23.5%) |
| Type of Diabetes | type I | 8 persons (24%) |
| | type II | 26 persons (76%) |
| Gene amplification | has it | 28 persons (82%) |
| | does not have | 5 persons (15%) |

**Table 2.** Patients and their clinicopathological information.