Mutational changes at *HPRT* gene locus in type-2 diabetes mellitus

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Abstract

Diabetes Mellitus (DM) is a metabolic defects of multiple etiologies characterized by disturbances of protein, fat and carbohydrate metabolism resulting from disorders in insulin secretion, insulin action or both. Studies of mutations at *HPRT* gene have provide insights into several aspects of somatic mutations *in vivo*, involving molecular mechanisms of mutagenesis, the relationship between DNA damage and mutation, as well as individual susceptibility factors like DNA repair capacity. In this study, somatic cell genetic marker; Hypoxanthine guanine phosphoribpsyltransferase (HPRT) was examined using short term lymphocyte culture in presence of 6-thioguanine (6-TG) as selective agent for HPRT on peripheral blood lymphocytes taken from twenty five type 2 DM patients using short term lymphocyte culture, their ages ranged between 30-80 years, and 15 healthy people with the same age range as control group.. Resistance to this selective agent refers to as genetic alterations at this HPRT locus. Lymphocytes taken from diabetic patients who were treated with 6-TG shows clear resistance to this analogue. Resistance to 6-TG in those diabetic patients is supported by high frequency of mutations fraction (MF) for those resistant cells. We conclude that type-2 diabetic patients have mutational changes at *HPRT* gene locus.
الخلاصة

مرض السكري هو مجموعة من الاضطرابات الأيضية الناتجة عن اسباب عدة ويتصف المرضى باضطرابات في أيضا البروتين والدهون والكربوهيدرات الناتجة من الاضطرابات في أفرز الانسولين أو عمله أو الالتحام معا. توفر دراسات الطفرات عند الموقع الجيني HPRT كوانين فوسفورايبوسيل ترانيغريز عدة اوجه للطرفات الجسدية داخل الجسم الحي متضمنة الآلية الجينية للتطغير وتوضح العلاقة بين ضرر المادة الوراثية والطفرة فضلا عن عوامل الاستعداد الفردية مثل قدرة إصلاح المادة الوراثية. استعملت الخلايا اللافاوية منذ فترة طويلة لتقنيم الضرر الذي يحدث على مستوى الجينات والذي قد يحصل داخل الجسم الحي فضلا عن خارجه.

جرى اختيار مؤشر وراثي لخلية الجسمية في الدراسة الحالية وهو (HPRT) باستخدام مزروع خلايا اللافاوية. قصير الأمد يوجد عامل انتخابي هو نظير الكوانين (6-ثايوكوانيين) الخاص بالمؤشر الوراثي الخلايا اللافاوية لعينات الدم المحلي لخمسة وعشرين مريضا مصابا من الذكور ولاتين بداء السكري النوع الثاني أستعمال مزروع الخلايا القصيرة الأمد. تراجعت اعماهم بين 30-80 سنة و15 فردًا من الأشخاص الاصحاء بمعدل الاعمار نفسها. تشير مقاومة الخلايا اللافوية إلى العامل الانتخابي إعلاء الى حدوث تغيرات وراثية عند الموقع الجيني HPRT. وما يدعم وجود مقاومة لدى هؤلاء المرضى للعامل الانتخابي الذي شملته الدراسة التردد العالي لعامل الطفور (MF) الخلايا اللافاوية المقاومة. نستنتج ان لمرضى السكري النوع الثاني تغيرات تطغيرية عند الموقع الجيني HPRT.
Introduction

Diabetes Mellitus (DM) is a metabolic defects of multiple etiologies characterized by disturbances of protein, fat and carbohydrate metabolism resulting from disorders in insulin secretion, insulin action or both (1,2). More than 20 regions in the human genome are associated with type 1 diabetes, but most make only a minor contribution overall to the susceptibility to type 1 diabetes (3,4). Large resources have been devoted to finding genes of type 2 diabetes. These effects have included many candidate gene studies and extensive efforts to fine-map linkage signals. Despite the detection of multiple genomic region linked to diabetes, linkage analysis and subsequent positional fine-mapping of candidates have been mostly indecisive (5). In a general population, the life time risk of type 2 diabetes is about 7%, 40% in offspring of one parent with type 2 diabetes, and about 70% if both parents have diabetes (6).

Hypoxanthine guanine phosphoribosyltransferase (HPRT) is a purin salvage enzyme which converts the purine bases hypoxanthine and guanine to the respective nucleotides inosine 5 monophosphate (IMP) and guanosine 5 mono phosphate (GMP) (7). Human genetic studies indicate that the structural gene for HPRT of this enzyme results in overproduction of uric acid leading to severe, precocious gout and nephrolethiasis (9). Complete deficiency of this enzyme causes Lesch-Nyhan syndrome containing of hyperuricemia, the hall marks of which are retardation, choreoathetosis and compulsive self mutation (9). Studies of mutations at HPRT gene have provide insights into several aspects of somatic mutations in vivo, involving molecular mechanisms of mutagenesis, the relationship between DNA damage and mutation, as well as individual susceptibility factors like DNA repair capacity (10). The resistance of 6-TG occurs by decreased HPRT activity required for activation of thiopurine to nucleotide form. Loss of HPRT activity is a consequence of point mutation or loss of the HPRT locus as a result of deletion or aberration containing the long arm of the X-chromosome where the gene located (11). We aim to assess mutational effects in the gene locus HPRT.
Materials and methods

Patients whom chosen for this study were men and women with diabetes mellitus disease. They have been chosen from various hospitals in Wasit province which include: Al-Zahraa Hospital, Al- Karama Hospital and Wasit Blood Bank. 25 patients with T2DM from two genders (males& females) with ages 30-80 years (mean age: 45.3 years). Fifteen healthy control groups similar to type 2 diabetes mellitus patients in age and gender were comprised in this study. Each patients and control groups were drawn about 2 ml of blood and putting into sterile vacutainer tube (10ml) containing 0.1ml heparin solution and was used for short-term cultures. Short-term peripheral blood lymphocyte cultures were done under optimal conditions. The samples of blood from patients and controls were cultured in RPMI-1640 culture medium (12). Preparation of RPMI-1640 culture medium was done under sterile conditions. 2.5 gram of RPMI-1640 was dissolved in 50ml of deionized distilled water (DDW), 2.5ml of antibiotic solution (streptomycin+ penicillin), 3.75ml of sodium bicarbonate solution, 2.5ml of bromodeoxyuridine (BUDR) solution and 25ml of fetal calf serum were added. The final volume was brought-up to 250 ml with deionized distilled water. Cultural medium sterilization was done by Millipore filter (0.22μm). The sterilized culture medium was kept at 4°C.

Culturing of peripheral blood lymphocytes was done using blood specimens obtained from healthy individuals. In these experiments, various concentrations of colcemide, PHA, in addition to various specimens of blood cultured were used to detect the optimization of the culture media and to obtain clear results. In this study, the standard blood culturing involved inoculation of 0.1ml of heparinized peripheral blood in 3ml of RPMI-1640 culture medium which was manipulated in 10 ml sterile vacutainer tube. Adding 0.2ml of PHA, then, incubated the culture medium at 37 °C for 72 hours (13). 6-TG powder was dissolved in sterilize deionized water to prepare the concentration 5 μ g/ml, and then kept at 4°C. Concentration of 6-TG was added to peripheral blood lymphocyte cultures for each sample of patients and controls to determine resistant cells (14). These cultures were incubated and harvested as previously mentioned with untreated cultures.
Cell harvesting method

Colcemide(0.1ml) in a concentration of 10 μg/ml was added to each culture tube for the final two hours of incubation time to harvest of mitogen stimulated cells (15). In the last of incubation time, tubes containing cells were centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and a little medium was remained over the cell pellet. 8ml of warmed hypotonic solution (37°C) was treated the harvested cells with gentle mixing, then, the tubes were incubated in a water bath at 37°C for 25 minutes with shaking every 5 minutes (13).

Growth Inhibition Calculation

Growth inhibition percent express the reduction in MI or BI of peripheral blood lymphocytes from diabetic mellitus and control groups treated with 6-TG, and was calculated according to the following formula: (14).

\[
\text{Growth inhibition} = \left( \frac{\text{Error}_{\text{BI}}}{} \right) \times 100 - 100
\]

Mutation Fraction Calculation

Mutation fraction measurement is the simplest way to calculate the mutation rate in presence of 6-thioguanine. The mutation fraction was calculated according to the following formula: (15)

\[
\text{MF} = \text{Error}
\]

* No. of lymphocytes was calculated by Differential method

** Total no. of white blood cells was calculated by WBC count standard method.

Results and discussion

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Table 1 shows the effect of 6-TG on percent of growth inhibition (%) of BI & MI of lymphocytes from DM, and control group. There was a cleared reduction in BI & MI parameters. The Blast index for lymphocytes of DM groups treated in vitro with 6-TG [19.79±4.31] which was significant lower than that of control group [35.33±1.98] (P<0.01). Similar result was seen in regard to mitotic index. Peripheral blood lymphocytes obtained from diabetic and control that treated with cytotoxic drug (6-TG) in vitro showed marked resistance to the analogue. This resistance represented by these lymphocytes ability to form blasts and dividing in the presence of the cytotoxic drug (6-TG). Resistance to 6-TG is reflected by high frequency of mutation fraction for these resistance cells. Studies of mutations at HPRT locus have provide insights into several aspects of somatic mutations in vivo, including the relationship between DNA damage and mutation and individual susceptibility factors such as DNA repair capacity (16).

Table 1: Effect of 6-TG on growth inhibition (%) represented by Diabetic Mellitus and control groups.

| Parameters       | 6-TG treated group |
|------------------|--------------------|
|                  | Control            | T2 DM               |
| Growth Inhibition % | BI 35.33±1.98     | 19.79±4.31          |
|                  | MI 64.4±6.93       | 44.8±4.91           |
| P-value          | 0.01**             | 0.01**              |

** significant different p-value 0.01

According to the values of MI, BI and growth inhibition of 6-TG treated patients, the results of this study indicate that to mutational alterations at HPRT gene locus.

The most commonly used genetic locus in cell mutation studies has been HPRT, the gene which codes for enzyme hypoxanthine guanine phosphoribosyltransferase. This enzyme is ubiquitous and catalyzes reactions involved with purine nucleotide salvage in mammalian cells.
It also binds and ribophosphorylates many toxic purine analogues including 6-thioquanine, which allows for in vitro selection of mutant T-cells that have acquired at HPRT mutation in vivo (17).

The hypoxanthine guanine phosphoribosyltransferase locus is reasonably easy to study it is X-linked and thus not masked by a second allele (18). The HPRT gene is an excellent biomarker of effect because mutations at the HPRT locus have no direct clinical consequences and have been shown to reflect genome-wide mutational events. An increase in frequency of mutation at this locus is commonly used as evidence of genotoxic insult and as a surrogate for genetic instability (19,20).

Evaluation Resistance to Cytotoxic Drug 6-TG in Diabetic Patients.

Blood cells taken from patients were cultured in vitro in presence of 6-TG, to select their resistance cells presents in circulation in vivo. To determine the incidence of such thioguanine resistance cells (TG R), we calculated the number of resistant cells per thousand which subsequently demonstrates the HPRT mutant cells in 6-TG treated cultures. According to 6-TG, the higher resistant cells have been observed was 78.94. Our results revealed to the presence of resistant cells to cytotoxic drug (6-TG) graduated from 18 in to 78.94. This indicates that DM patients showed resistance to 6-TG, hence presence of mutagenesis changes at HPRT gene locus (Table 2).
Table 2: Resistant cells of peripheral blood lymphocytes (RC x10^3) treated with 6-TG from patients with diabetic group.

| Patient No | Resistant cells | Patient No | Resistant cells | Patient No | Resistant cells |
|------------|-----------------|------------|-----------------|------------|-----------------|
| 1          | 18.75           | 10         | 37.03           | 19         | 75              |
| 2          | 54.75           | 11         | 25              | 20         | 14.61           |
| 3          | 50              | 12         | 18.69           | 21         | 49.01           |
| 4          | 40.38           | 13         | 27.34           | 22         | 15.30           |
| 5          | 37.5            | 14         | 8.82            | 23         | 3.64            |
| 6          | 21.73           | 15         | 31.15           | 24         | 36.60           |
| 7          | 21.34           | 16         | 23              | 25         | 46.80           |
| 8          | 32.96           | 17         | 78.94           |            |                 |
| 9          | 42.1            | 18         | 28.14           |            |                 |
| Mean ± SE |                 |            |                 |            | 33.54±4.08      |

According to our results there is an important relation between genetic alteration at HPRT locus, in that, folic acid deficiency promotes instability in human DNA, causing damage as increased chromosomal abnormalities and elevation mutant frequencies in the HPRT gene. Supplementation of folic acid can decrease or reverse these effects (21,22).

We conclude that type-2 diabetic patients have mutational changes at HPRT gene locus.
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