Establishment of *HPRT* and *DHFR* Gene Mutation Assays as biomarkers In Sheep lung fibroblasts (SLF)

فحص الطفرة الوراثية لجين الـHPRT و *DHFR* كمؤشرات وراثية في خلايا رئة الاغنام

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Abstract

The aim of this study is to establish a gene mutation assay for examining the integrity of animal cell genome for nuclear transfer technique. Lung fibroblasts which were expanded from 4 months old female lamb were selected as target cells. These cells were coded (SLF) as Sheep lung cells. Growth characterization, doubling time, chromosomal number and structural integrity were checked after their growth in RPMI-1640 medium. For *HPRT*-gene mutation assay, the cells were plated at density of 1×10^3 cells/plate and grown in medium containing toxic concentrations of 6-thioguanine; while for *DHFR* -gene mutation assay, toxic concentration of methotrexate was used as a selective agent. Those cells were grown for 15 days; mutant colonies either $6TG^r$ or MTX^r were reinoculated in a selective medium for further 8 weeks for checking the stability of phenotypic expression of mutant cells. The results revealed that SLF cells has spontaneous frequencies of *HPRT*, and *DHFR* gene mutations equal to 16 and 22×10^{-3} event/ generation/ cell, respectivel.These levels are normal comparing with other animal cell types, and these assays could be applied on other somatic cells.

المستخلص

كان الهدف من هذه الدراسة هو ايجاد طريقة Biomarker لفحص الطفرات الوراثية لجين الـHPRT والـ *DHFR* كمؤشرات حيوية تفيد في فحص سلامة الذخيرة الوراثية للخلايا الجسمية المراد استعمالها في عمليات نقل الانوية . استعملت خلايا رئة الاغنام(SLF) sheep lung fibroblasts التي سبق وان عزلت من رئة انثى حمل بعمر 4 اشهر ، وتمت تنميتها مختبرياً لمدة عام كامل في مختبرات منظمة الطاقة الذرية . درست خصائص النمو، زمن التضاعف والهيئة الكروموسومية التركيبية والعددية للخلايا بعد تنميتها في الوسط الزرعي (RPMI-1640) . ولغرض فحص التردد التلقائي للطفرة الوراثية لجين الـ HPRT ، تمت زراعة الخلايا بعدد 1000 خلية/طبق وفي وسط زرعي حاوي على التركيز القاتل من نظير الدكوانين -6) (6TG Thioguanine ولمدة 15يوماً . بينما تمت تنمية الخلايا للفترة ذاتها في وسط زرعي حاوي على التركيز القاتل من مادة الـ(MTX)Methotrexate لغرض الكشف عن التردد التلقائي للطفرة لجين *DHFR* ولغرض التأكد من ظهور فعل الجين وثبوتية الطفرة ، احتسبت المستعمرات الطافرة لكل جين وتمت اعادة زراعتها في الوسط الزرعي المنتخب لكل طفرة ولمدة 8اسابيع . تشير النتائج إلى ان خلايا رئة الاغنام المستعملة في هذا البحث تحوي على مستوى تلقائي من الطفرة في جين HPRTوDHFR تساوي 16 و22 × 10^{-3} حدث/جيل/الخلية . وهو مساوي إلى ذلك المكتشف في خلايا حيوان ألهامستر المستخدمة في المختبرات عالميا . يمكن تطبيق هذه التقنية على خلايا جسمية اخرى .

Introduction

In somatic cell genetics studies, markers which have received considerable attention in mutagenesis, including: loci for hypoxanthine guanine phosphoribosyl transferees (HPRT), adenine phosphoribosyl transferees (APRT), thymidine kinas (TK) and dihydrofolate reeducate (DHFR). HPRT is a purinic salvage enzyme which converts the purine bases hypoxanthine and guanine, to their respective nucleotides IMP and GMP. Although, the enzyme is not required for cell growth, agents such as aminopterin can be used to block *de novo* synthesis of nucleotides thus making cellular multiplication dependent on the presence of HPRT [1]. HPRT deficiency is the biochemical defect in human Lesch-Nyhan syndrome and its gene has been located on X-chromosome [2].

Dihydrofolate reductase (DHFR) catalyses the reduction of 7.8- dihydrofolate (DHF) to 5,6, 7,8- tetrahydrofolate (THF), by the coenzyme NADPH [3]. DHFR enzyme is a paramount significance not only because it is necessary for THF formation, but it coupled with thymidylate synthase (TS), it is essential for thymidylate production through the *de novo* pathway [4]. This enzyme is, therefore, pivotal in the production of purines and pyrimidines precursors for RNA, DNA and amino acid biosynthesis at various stages of cell survival or cycling. The DHFR enzyme levels are highly elevated due to gene amplicfication in the chemically (such as methotrexate) or virally transformed cells as well as malignant cells [5]. *DHFR* gene and enzyme is a biomarker for highly proliferative cells, thus it has studied for long as a target for antiproliferative agents due to its crucial role in DNA synthesis and its mechanistic features [6, 7]. Genetic studies have shown that *DHFR* gene is located on chromosome 5 at 11.1- q 13.2 of animal and human cells [8]. In the last two years, we had successfully isolated and explant normal lamb lung fibroblasts. This study is conducted to establish mutation assays for *HPRT* and *DHFR* genes in normal lamb lung cells as biomarkers for genome stability and integrity tests.

Materials and Methods

Cells

The target cells were established from sheep lung fibroblast (SLF) in the laboratory of Animal Health Department Directorate of Agriculture and Biology, from 4-month old female lamb.

SLF cells were grown exponentially in RPMI-1640 medium which was supplemented with 10% fetal calf serum and penicillin: streptomycin (100 IU/100 μg/ml) for 48hr. colchicines at final concentration of 10μg/ml was added to arrest the cells at metaphase in the final 4 hrs. of incubation period. Air-dried slides were prepared from those cultures and stained with Giemsa for chromosomal analysis [9]. The mitotic index and the metaphase with 54 chromosomes were determined using blood lymphocyte as model cells [10].

Plating efficiency (PE):

Exponentially growing SLF cells in RPMI- 1640 medium were inoculated 100, 200, 300,500 and 1000 cells for determination of colony forming ability. The cultures were incubated for 2 weeks at 37°C at 5% CO_2. The growing colonies were rinsed in buffer

saline and fixed with methanol: acetic acid (3:1 v:v) for 10 minutes, rinsed and stained with 2.5% Giemsa solution for 30 min then rinsed and dried.

The number of colonies was counted as [11]:

$$PE = \frac{Colonies}{Inoculated\ cells} \times 100$$

Cellular growth and doubling time

For drawing the growth curve and determining the doubling time, exponentially growing SLF cells were inoculated at 10^3 cell / T25 flasks in RPMI-1640 medium and incubated for (24, 48, 72, 96, 120, 144)hrs. At each time, the cells were harvested with trypsinization and the viable cells were counted using try pan blue method [12].

Gene mutation

1. *HPRT*- Locus: The frequency of gene mutation at *HPRT* locus in SLF cells was determined as described for other cells [13]. By seeding the exponentially growing cells at different 6-thioguanine concentrations (10, 20, 30 and 40) μg/ml for 15 days. The numbers of 6TG^r- colonies were counted.

    Phenotypic stability of HPRT-mutation was checked by growing the cells for 8 weeks in 6TG medium.

2. *DHFR*- locus: Spontaneous mutation at *DHFR* gene in SLF cells was checked by inoculating the exponentially growing cells into methotrexate (MTX) containing medium at concentration of MTX of (2.5, 5.0 and 10) μg/ml, for 2 weeks. Phenotypic expression of MTX^r- mutation was checked by continuous growing of mutant colonies in MTX-medium at concentration of 10 μg/ml up to 8 weeks.

Statistical analysis

Data were analyzed by factorial analysis of variance using least sequare analysis method [14].

Results and Discussion

Cytogenetic analysis of sheep lung fibroblasts showed a chromosomal number of 54 with no chromosomal aberrations could be seen. In this investigation, the results presented in Table (1) showed that the average cloning efficiency of sheep lung fibroblasts in RPMI-1640 medium grown for 15 days was 51.3 ±1.3. This potency was different from that of human fetal lung fibroblasts (IMR-90) which had a plating efficiency about 35% [15], or Chinese hamster lung fibroblasts (V-79) cells with a plating efficiency of 90% [16]. These variations might due to species differences [17].

Table (1): Plating efficiency of sheep lung fibroblasts.

Number of seeded cells	Number of growing colonies	Plating Efficiency PE (%)
100	52	52.0
200	96	48.0
300	147	49.0
500	262	56.0
1000	515	51.5

Average plating efficiency = 51.3 ±1.3

Sheep lung cells had grown exponentially from the time of inoculation up to 5th day with growth ratio of 15.46; post that time the decline in the growth showed up. The doubling time of those cells was estimated to be 10-16 hrs. Table (2).

Table (2): Growth performance of sheep lung fibroblasts in RPMI-1640 Medium for 7 days.

Period of incubation (hr)	Number of growing cells	`growth ratio
0	1500	-
24	3200	2.13
48	5600	3.73
72	11800	7.86
96	23200	15.46
120	23200	15.46
144	11600	7.73

Optimum growth was ranging between day 4th-5th post inocubation.

These cells different from that of sheep blood lymphocytes [18], and goat blood cells [19] which had doubling time 16-18hrs, while V79 cells had doubling time with range of 10-12 hrs. [16]. These differences within the species in doubling time might due to deference between established cell lines from primary cultured cells even they grow under similar medium and other growth conditions [20].

Table (3):* Spontaneous Mutation fractions of *HPRT* AND *DHFR* genes in sheep lung cells.

Concentration of the selective agent (µg/ml)	No. of seeded cells/plate	No.of colonies /plate	Cloning efficiency ** (%)	Mutation fraction event/generation /cell
6-Thioguanine				
0	1000	480	94.1	-
10	=	064	66.6	54.5×10^{-3}
20	=	028	29.1	33×10^{-3}
30	=	016	16.0	16×10^{-3}
40	=	000	0.0	0.0
Methotrexate				
0	1000	492	96.4	-
2.5	=	85	17.2	85×10^{-3}
5.0	=	59	11.9	59×10^{-3}
10.0	=	22	4.4	22×10^{-3}

* Data presented as an average of three independent experiments
** Counted as a percentage of controls

In this investigation, it was necessary to determine the concentration of the selective agent for the selection of mutant cells. High concentration of 6 thioguanine (40 µg/ml) was found to reduce completely cellular replication Table (3).

However, at 20 µg/ml of this analogue, only the spontaneously mutated cells with a mutation fraction of 28×10^{-3} events/generation/ cell were survived. These mutant cells were grown normally with a phenotypic expression of 6TG-resistance for more than 8 weeks in 6TG-medium. Further increase of 6TG concentration to 30µg/ml was significantly reduced cellular viability down to PE of 16% (i. e with mutation fraction of 16.0×10^{-3}). The causes of such reduction are unknown. 6TG was found to induce chromatid damage [21] and arrest the cells at S-phase by inducing unrepaired DNA lesion [22]. This analogue was found to inhibit completely the cellular division and

replication by days of treatment of human lymphoblastoid and leaving the resistant cells to replicate normally. Cells resistant to 6-TG were found to have no HPRT enzyme activity [3, 23, 24]. Thus 6 TG-resistant sheep lung cells have a spontaneous mutation at *HPRT* gene as other cells with fraction of 33×10^{-3} at a concentration of 20 µg/ml of 6TG and 16×10^{-3} at a concentration of 30 µg/ml Table (3).

The results of SLF cell cloning in methotrexate medium are showed in Table (3). MTX -resistant cells had a plating efficiency of 17.2% of the wild type at 2.5 (µg/ml) of MTX. however, these were reduced to 4.4% at the 10(µg/ml) of this antifolate drug. The mutation fraction of MTX-resistance was 22×10^{-3} (events/gene ration /cell) at 10 µg/ml of MTX..The phenotypic expression of these mutations was stable when the cells were grown for more than 8 weeks in MTX-medium.

The events that lead to the cytotoxic action of MTX begin with drug movement across cellular membrane, followed by its intracellular metabolism to the polyglutamate derivative, binding to dihydrofolate reductase (DHFR) and other folate dependent enzymes and finally initiating DNA synthesis.The possibility that naturally occurring DHFR alleles may exist in a variety of tissues was reported [25]. The *DHFR* genetic polymorphism may serve as a mechanism by which the cells may become MTX-resistant (i.e. amplification of MTX insensitive alleles). In addition to gene amplification ,more subtle mechanisms exist for increasing DHFR expression in the presence of MTX [26].Thus the mechanisms of MTX-resistance includes *DHFR* gene mutation ,amplification of target gene, altered drug transport, differences in nucleosides and nucleobase salvage pathway [27].

These results suggested that SLF cells have spontaneous *DHFR* gene mutation (gene amplification). Within normal levels as in other cells [16] further investigation is required for determination of the *DHFR* enzyme activity in different MTX-resistant cells.

Thus we achieved the establishment of *HPRT* and *DHFR* gene-mutation assay as biomarkers for checking genome stability of animal cells for nuclear transfer techniques.

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