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Genistein Induced Hemizygous-Type Loss of Heterozygosity in Thymidine Kinase Gene of L5178Y Cells

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Abstract: This aim of this was to evaluate the mutagenic effect and mechanisms of genistein. The mutagenic effect of genistein was assessed by the mouse lymphoma assay in L5178Y cells treated with genistein for 3 and 24 h. To investigate the characteristics of mutagenesis, the specific mutation point of Thymidine Kinase (*TK*) gene was identified by DNA sequencing and analyzed by Real-Time quantitative PCR (qRT-PCR). The Total Mutation Frequency (TMF) increased from 59.07×10^{-6} to 1038.47×10^{-6} in a dose-dependent manner after genistein exposure. The difference in the TMF between the vehicle control and genistein groups (5, 10 and 20 μg mL^{-1}) was statistically significant ($p<0.01$). DNA sequencing identified an overlapping-peak (T/G) heterozygote at the 64th base of exon6 in the *TK* gene. By comparison in the induced and spontaneous mutant colonies, a single-peak (G) existed at the same position which was the reflected Loss of Heterozygosity (LOH). The qRT-PCR results showed that the proportion of hemizygous LOH in spontaneous mutant colonies was 29.03% which increased to 84.38% in genistein-induced mutant colonies. Genistein (5-20 μg mL^{-1}) induced TK mutations in a dose-dependent manner in L5178Y cells and the mutations were mainly hemizygous-type LOH in *TK* gene.

Key words: Genistein, mouse lymphoma assay, DNA sequencing, real-time quantitative-PCR, mutagenicity, loss of heterozygosity

INTRODUCTION

Genistein is a phytoestrogen discovered in soybean seeds. Epidemiology and animal experiments demonstrate that genistein has various potential physiological activities that prevent many diseases including breast and prostate carcinoma, cardiovascular diseases and post-menopausal diseases (Barnes, 2010; Engel *et al*., 2012; Gencel *et al*., 2012; Weng *et al*., 2013). Foods containing phytoestrogens are found to be beneficial to human health. Researchers in different fields commonly focus on these compounds and some phytoestrogens have been clinically applied. However, phytoestrogens also have side effects that may be potentially harmful to human health. Genistein may reportedly cause reproductive and developmental toxicity (Rozman *et al*., 2006), genetic toxicity (Stopper *et al*., 2005) and risk of cancer induction (Taylor *et al*., 2009). Therefore, the mutagenic effect of genistein should be investigated. The Mouse Lymphoma Assay (MLA) is an *in vitro* mammalian mutation assay used to screen possible carcinogens and mutagens (Ogawa *et al*., 2009). MLA uses the Thymidine Kinase (*TK*) gene of the L5178Y/$TK^{+/-}$-3.7.2C mouse lymphoma cell line as a mutation reporter gene and can identify not only point mutations and small deletions but also large deletions, translocations, recombination and many different types of chromosomal mutations (Wang *et al*., 2009; Lloyd and Kidd, 2012). DNA sequencing is a molecular method commonly used to obtain DNA sequence information. In Real-Time quantitative PCR (qRT-PCR), fluorescence groups are added to a PCR system and PCR progression is monitored by a detecting fluorescent signal. Then, gene dosage is quantified using a real-time Cycle threshold (Ct) value. In the present study, the mutagenic effect of genistein was detected by MLA. L5178Y cells were used and these cells were initially treated with toxins for 3 h. However, the 3 h treatment was insufficient for certain clastogen and spindle poisons and a risk of obtaining false negative data existed. To study further the genotoxic effect of genistein, this study compared the mutagenic effect of genistein between the 3 and 24 h treatment groups.

MATERIALS AND METHODS

Reagents: Genistein was purchased from Sigma (St. Louis, MO, USA) and dissolved in Dimethylsulfoxide (DMSO). The purity of genistein was >99%. Trypsase was

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obtained from Gibco (Grand Island, NY, USA). Thymidine (T), Hypoxanthine (H), Methotrexate (MTX), Glycine (G), Trifluorothymidine (TFT), Mitomycin C (MMC), Sodium pyruvate and vitamin C were purchased from Sigma (St. Louis, MO, USA).

Cell culture: L5178Y $TK^{+/-}$-3.7.2C cells were provided by Masamitsu Honma from the Division of Genetics and Mutagenesis, National Institute of Health Sciences, Japan. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated horse serum (Gibco), 200 μg mL^{-1} sodium pyruvate, 100 unit mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and maintained at densities ranging from 10^5-10^6 cells mL^{-1}.

Dosage of genistein: The preliminary experiment showed that genistein decreased the Relative Suspension Growth (RSG) of L5178Y cells in a dose-dependent manner. RSG was between 20 and 40% when the cells were treated with 20 and 10 μg mL^{-1} genistein for 3 and 24 h, respectively, suggesting significant cytotoxicity. In most cases, RSG was greater than the Relative Survival (RS) which ideally ranges from 10-20%. Hence, treatments of the cells with 2.5, 5, 10 and 20 μg mL^{-1} genistein for 3 h as well as with 1.25, 2.5, 5 and 10 μg mL^{-1} genistein for 24 h were deemed optimal. The cells in the positive control, solvent control and negative control groups were treated with 0.5 μg mL^{-1} MMC, 0.5% DMSO and purified water, respectively.

Mouse Lymphoma Assay (MLA): Spontaneous mutant cells were removed prior to the experiment. To reduce the background mutant fraction, cells were incubated for 24 h in RPMI1640 plus THMG (3 μg mL^{-1} thymidine, 5 μg mL^{-1} hypoxanthine, 0.1 μg mL^{-1} MTX and 7.5 μg mL^{-1} glycine) before the experiments. The cells were then incubated in the same medium without MTX for 48 h. Cells were treated with different doses of genistein for 3 and 24 h. After treatment (day 0), cells were washed twice with PBS and resuspended in fresh culture medium, gradient diluted into 8 mL and cloned in 96-well plates with 200 μL per well. The plates were cultured for 12 days and visually evaluated to determine the presence or absence of a colony in each well. Then, the plating efficiency on day 0 (PE_0) was calculated. The remaining cells in day 0 were cultured for 2 days to express the mutant phenotype and the plating efficiency on day 2 (PE_2) was determined. The density of cells on day 2 were adjusted to 1×10^4 mL^{-1} and cloned in 96 well plates with 200 μL per well in a medium containing TFT (Final concentration = 3 μg mL^{-1}). After 12 days of incubation, the mutant colonies in 96 well plates were evaluated and the Total Mutation Frequency (TMF) was calculated. The methods for calculating RS, RSG, Relative Total Growth (RTG) and TMF are described elsewhere. The data were statistically analyzed using Mutant TM.

Screening of mutant colonies: Mutant colonies were initially selected from thehighest-dose group treated with genistein for 3 h and the negative control group. Cells were cultured in a 24 well plate and then in a 6 well plate. All colonies were cultured in a medium containing TFT or THMG. Colonies that grew in TFT medium and did not grow in THMG medium were used in subsequent experiments.

DNA sequencing: RNA was extracted from mutant colonies. cDNA was obtained using a Revert Aid™ First-strand cDNA Synthesis Kit from Fermentas. The length of *TK* gene cDNA in a mouse lymphoma cell is 1389 bp and its coding sequence ranges from 236-937 bp. A 947 bp long fragment ranging from 119-1066 bp was obtained using the primers for RT-PCR and the sequence was further sequenced. The RT-PCR primer was as follows: TK (947 bp), 5'-TGGGACGAGTCTTGTCTT-3' (sense) and 5'-GAACTGA AAACGGCTTCAG-3' (antisense). The cDNA products were harvested using a 0.5 mL Eppendorf tube and sequenced by the Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The primer for sequencing was TKSF, 5'-GGCAAATGCGAGCAG TAAGT-3'.

qRT-PCR: Genomic DNA was extracted and qRT-PCR was performed using a FTC2000 fluorescence qRT-PCR apparatus. A pair of primers flanking the 64th base of exon6 can be used to amplify a 95 bp long fragment. Moreover, a fluorescent probe was inserted into the 23rd base upstream of the 64th base. A 123 bp long DNA fragment of VEGF was used for normalization as the internal reference gene. The PCR primers were as follows: VEGF (123 bp), 5'-GTGATCAAGTTCATGGATGTCTA-3' (sense) and 5'-GCCGTCCTG TGTGCCGCTG-3' (antisense); and TK-FQ (95 bp), 5'-TCGGC AGCATCTTGAACCT-3' (sense) and 5'-AGAAGCTGCCTACACGAAG-3' (antisense). The probe sequences were as follows: VEGF, 5'-CCCTGGTGGACATCTTCCAGG-3' and TK-FQ, 5'-GTGGT GAAGCTCACCGCTG-3'.

Data analysis: The relative expression of the target gene (*TK*) was obtained by the following equation:

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

Ct represents the intensity of the fluorescent signal and the amplified efficiency of the target gene was equal to the amplified efficiency of housekeeping gene. ΔΔCt = [Ct TK (target sample)-Ct VEGF (target sample)]-[Ct TK (regulation sample)-Ct VEGF (regulation sample)]. The expression of the regulation sample was set to one and the regulation sample was the TK expression in normal L5178Y cells. Hence, $2^{-\Delta\Delta Ct}$ represents the relative expression of the target gene. The detected mutation site was a hemizygous-type Loss of Heterozygosity (LOH) when the ratio reached approximately 0.5 and a homozygous-type LOH when the ratio reached approximately 1.0.

RESULTS AND DISCUSSION

Detection of the mutagenicity of genistein: When cells were treated with genistein for 3 h, RS, RSG and RTG decreased. However, TMF increased in a dose-dependent manner. As shown in Table 1, a statistically significant difference was observed between the TMF of the vehicle control and 3 h genistein-treated groups at 5, 10 and 20 μg mL^{-1} ($p<0.01$). Furthermore, in cells treated with genistein for 24 h, RS, RSG and RTG decreased whereas TMF increased in a dose-dependent manner. Besides, there was a statistically significant difference in between the TMF of the vehicle control and 24 h genistein-treated groups at 5 and 10 μg mL^{-1} ($p<0.01$). No statistical significance was observed for the TMF of *TK* gene between the 3 and 24 h groups ($p>0.05$).

DNA sequencing: In this study, 71 samples were analyzed by DNA sequencing. These samples contained 35 genistein-induced mutant colonies (17 small and 18 large colonies), 35 spontaneous mutant colonies (17 small and 18 large colonies) and normal L5178Y cells. Homology comparison analysis showed that T and G simultaneously presented at the 64th base of exon6 of *TK* gene in normal L5178Y cells. However, the same site in all mutant colonies was represented only by G. Sequencing results showed that two peaks presented at the 64th base of exon6 of *TK* gene in L5178Y cells. By comparison, the same site in the 35 induced mutant colonies and 35 spontaneous mutant colonies was single peak which reflected LOH. qRT-PCR results: A total of 31 spontaneous mutant colonies (16 small, 15 large) and 32 induced mutant colonies (15 small, 17 large) were analyzed by qRT-PCR. Results demonstrated that 5 of 16 (31.25%) small spontaneous mutant colonies were hemizygous LOH, 4 of 15 (26.67%) large spontaneous mutant colonies were hemizygous LOH, 13 of 15 (86.67%) small genistein-induced mutant colonies were hemizygous LOH and 14 of 17 (82.35%) large genistein-induced mutant colonies were hemizygous LOH. The proportion of hemizygous LOH in spontaneous mutant colonies was 29.03% which increased to 84.38% in genistein-induced mutant colonies (Table 2).

The plant estrogen genistein has been comprehensively used as an alternative therapy for multiple diseases, especially for hormone substitution in menopausal women (Hertrampf *et al*., 2007). Long-term treatment with genistein results in a number of side effects and many studies have reported its potential genetic toxicity. In this study, the mutation frequency in L5178Y cells increased when the genistein concentration was >5 μg mL^{-1} suggesting that a larger dose of genistein may have a mutagenic effect. McClain *et al*. (2006) found through *in vitro* MLA that genistein can increase the mutant frequency of *TK* gene with and without metabolic activation (S9). Di Virgilio *et al*. (2004) reported the generation of micronuclei in response to genistein treatment. Morris *et al*. (1998) observed that genistein increased the mutation frequency of AHH-1 $TK^{+/-}$ ($p53^{+/-}$) cells but showed no obvious effect on L3 ($p53^{+/+}$) cells, suggesting that the mutagenic effect of genistein depended on the p53 state. The MLA was used to evaluate the mutagenic effect of genistein. According to OECD Guidelines for Testing of Chemicals (No. 476, 1997), the mutagenic effect of the test subject should be evaluated by the MLA with and without S9. However, 3 and 24 h treatments of genistein were compared and some molecular biology methods were used to explore the mechanism of the mutagenesis of genistein by detecting its mutagenic effect; accordingly, only the experiment without S9 was

Table 1: Detection of genistein-induced mutagenicity

Groups	RS (%)	RSG (%)	RTG (%)	TMF($\times10^{-6}$)
Negative control	100.00	100.00	100.00	31.17
Solvent control	72.70	45.00	32.72	60.69
3 h treatment				
2.5 μg mL^{-1}	77.16	79.80	61.58	108.74
5.0 μg mL^{-1}	74.91	77.30	57.90	241.90*
10.0 μg mL^{-1}	47.56	64.80	30.82	758.74*
20.0 μg mL^{-1}	20.20	23.10	4.67	1038.47*
24 h treatment				
1.25 μg mL^{-1}	66.34	62.73	41.61	59.07
2.5 μg mL^{-1}	49.30	54.08	26.66	130.25
5.0 μg mL^{-1}	45.86	50.53	23.17	201.53*
10.0 μg mL^{-1}	25.89	37.64	9.75	705.62*
MMC (0.5 μg mL^{-1})	15.26	38.73	5.91	961.45*

*Comparison with the solvent control group, $p<0.01$

Table 2: Results of fluorescence quantitative RT-PCR

Groups	No. of sample	Homozygous (%)	Hemizygous (%)
Spontaneous mutation	31	22 (70.97%)	9 (29.03%)
Small colonies	16	11 (68.75%)	5 (31.25%)
Large colonies	15	11 (73.33%)	4 (26.67%)
Genistein	32	5 (15.63%)	27 (84.38%)
Small colonies	15	2 (13.33%)	13 (86.67%)
Large colonies	17	3 (17.65%)	14 (82.35%)

performed. False negative results may occur when cells are treated with clastogen or spindle toxins for only 3-6 h. Earlier studies have shown that the mutation frequency of *TK* gene in cells treated with diethylstilbestrol, hydroquinol, NSC-3096 and leurocristine for 24 or 48 h is higher than in cells treated for 3 h by the MLA and micronuclei assays (Honma *et al*., 1999a, b). These findings suggest that extending the treatment time is critical to the detection of the mutagenicity of aneuploid inducer and spindle toxins. In another study wherein cells are treated with toxins for 24 h, 11 of 15 negative or unclear results in the 3 h treatment group are positively detected, including base analog, nucleic acid analog and spindle toxins (Honma *et al*., 1999a, b). This finding further confirms that a 3 h treatment may lead to false negative data in the MLA assay therefore, a longer treatment may significantly increase the detection rate. In the present study, no obvious statistical significance for the mutation frequency of TK were observed between the 3 and 24 h treatment groups, suggesting that genistein was not a spindle toxin and that its genetic toxicity was not enhanced by extending the treatment time. Compared with other *in vitro* short-term genetic toxicity detection methods, the MLA can detect various genotoxic effects including point-mutation, deletion, recombination, aneuploid mutation and chromosome aberration. The *TK* gene of L5178Y cells is positioned on the distal E1-E2 of the long arm of chromosome 11 with a length of 14.5 kb. A study on the *TK* gene sequence shows that the point mutations on *TK* gene enable it to have functional and non-functional alleles. At the same time, the existence of multiple microsatellite regions (each having a number of different $(AC)_n$ repeat sequences) provides a theoretical basis for the study of *TK* gene mutations (Fellows *et al*., 2011). In the present study, the cDNA of TK in L5178Y cells was amplified using RT-PCR and then sequenced. The sequenced results of normal L5178Y cells showed that a two-peak heterozygote at the 64th base of the 6th exon of *TK* gene. T and G bases were simultaneously present at this site which indicated that *TK* gene had functional and non-functional alleles. By comparison, the mutated colonies at the 64th bases of the 6th exon of *TK* gene were found to be single peak, suggesting that the mutation was an LOH. Honma *et al*. (2001) found that LOH is a major mutational event in both spontaneous and induced mutations and divided the gene mutations of TK into seven categories: gene mutations, deletions, homologous recombination, gene conversion, illegitimate recombination whole functional chromosome loss and whole functional chromosome loss accompanied by a functional chromosome replication. Deletion, illegitimate recombination and whole functional chromosome loss are hemizygous LOH; homologous recombination, gene conversion and whole functional chromosome loss accompanied by a functional chromosome replication are homozygous LOH. The mechanism of genistein-induced LOH is largely unknown hence, the site was quantitatively analyzed by qRT-PCR. The threshold method was used for real-time PCR analysis. The qRT-PCR was used to detect the ratio of gene expression of mutant colonies to normal cells. The hemizygous-type LOH presented at a ratio of 0.5 and the homozygous-type LOH presented at a ratio of 1.0. The data in the current study showed that the proportion of homozygous-type LOH in spontaneous mutant colonies was 70.97%, indicating that the mutation at the 64th base of exon6 in spontaneous mutant colonies may be mainly homozygous-type LOH which may be caused by gene conversion. The generation and stability of spontaneous mutation is influenced by many factors such as the accuracy of DNA replication, the spontaneous chemical reaction of DNA bases, the capability of the mutation repair system and others. In the replication progress of DNA, the replication components often form insertion error and sliding error which are more easily converted to base substitution in the next round of replication. Genistein can reportedly disturb the function of topoisomerase II in cells (Schmidt *et al*., 2008). Topoisomerase II is a basic enzyme widely present in numerous eukaryotic and prokaryotic organisms. The enzyme is involved in DNA replication, transcription, recombination, repair and others, playing an important role in the process of life. Therefore, given that genistein is an inhibitor of topoisomerase II, treatment with genistein may damage DNA. Ouyang *et al*. (2009) found that 10-100 mM genistein treatment can lead to DNA Double-Strand Breaks (DSBs) in HO-8910 cells. Fox *et al*. (2012) also found that treatment with genistein can result in a significant induction of DSBs. In the present study, the proportion of hemizygous-type LOH in spontaneous mutant colonies was 29.03% which increased to 84.38% in genistein-induced mutant colonies.

CONCLUSION

This finding suggested that the mutation at the 64th base of the 6th exon in genistein-induced mutant colonies may be hemizygous-type LOH which may be due to a genistein induced DNA strand break that finally led to deletion in *TK* gene. The mutagenicity of genistein was evaluated by the MLA. Genistein (5-20 μg mL^{-1}) increased TMF in L5178Y cells in a dose-dependent manner. However, its effect did not depend on the treatment time suggesting that it did not present spindle toxicity. DNA sequencing indicated that genistein can induce LOH. The mutation site was analyzed by qRT-PCR. Results suggested that the spontaneous mutation in L5178Y cells may be mainly homozygous-type LOH and that the genistein-induced mutation may be hemizygous-type LOH.

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