

RESEARCH ARTICLE

No Effect of High Fat Diet-Induced Obesity on Spontaneous Reporter Gene Mutations in *gpt* Delta Mice

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Abstract

A large number of epidemiological studies have demonstrated that obesity is a risk factor for several human cancers. Several animal studies using rodents with diet-induced or genetic obesity have also demonstrated that obesity can promote tumor development. However, the effects of obesity on the early stages of carcinogenesis, and especially on the spontaneous occurrence of somatic gene mutations, remain unclear. To investigate the effects of obesity on the rate of spontaneous gene mutations, we performed reporter gene mutation assays in liver, kidney, and colon, organs in which obesity appears to be associated with cancer development on the basis of epidemiological or animal studies, in mice with high fat diet (HFD)-induced obesity. Six-week-old male and female C57BL/6 *gpt* delta mice were fed HFD or standard diet (STD) for 13 or 26 weeks. At the end of the experiments, reporter gene mutation assays of liver, kidney, and colon were performed. Final body weights and serum leptin levels of male and female mice fed HFD for 13 or 26 weeks were significantly increased compared with corresponding STD-fed groups. Reporter gene mutation assays of liver, kidney, and colon revealed that there were no significant differences in *gpt* or *Spi*ⁱ mutant frequencies between STD- and HFD-fed mice in either the 13-week or 26-week groups. These results indicate that HFD treatment and consequent obesity does not appear to influence the spontaneous occurrence of somatic gene mutations.

Keywords: Obesity - *in vivo* mutagenicity

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Introduction

A large number of epidemiological studies have demonstrated that obesity is a risk factor for several human cancers. A previous systematic review and meta-analysis showed that a 5 kg/m² increase in body mass index (BMI), commonly used as a marker of body fatness, is strongly associated with increased risk of several human cancers including colon [risk ratio (RR) 1.24] and renal cancers (RR 1.24) in men, and renal cancer (RR 1.34) in women (Renehan et al., 2008). A greater BMI is also a modifiable risk factor for colon cancer for Asia population (Morrison et al., 2013). On the basis of epidemiological studies, the World Cancer Research Fund and the American Institute for Cancer Research suggested that greater body fat is associated with increased risk for several cancers, including colorectal, kidney, pancreas, breast (postmenopausal) and endometrial cancer, and has been suspected as a causative factor for gallbladder cancer. In addition, there is limited evidence suggesting that greater body fatness increases the risk of liver cancer (World Cancer Research Fund, 2007).

Several animal studies using rodents with diet-

induced or genetic obesity have also indicated that obesity has the potential to promote tumor development. Excessive feeding of dietary fat such as corn oil has been reported to promote azoxymethane (AOM)-induced colon carcinogenesis in F344 rats (Reddy and Maeura, 1984). The *db/db* mouse, a genetically altered animal model with obesity due to a functional defect in the long-form leptin receptor (Lee et al., 1996), is sensitive to chemical carcinogenesis such as AOM-induced colon carcinogenesis (Hirose et al., 2004) and diethylnitrosamine (DEN)-induced liver carcinogenesis (Iwasa et al., 2010). In previous studies, various pathophysiological mechanisms linking obesity to cancer have been suggested. Insulin resistance and subsequent abnormal activation of insulin-like growth factor (IGF) and the IGF-1 axis has been considered to play a key role in carcinogenesis (van Kruijsdijk et al., 2009; Shimizu et al., 2011). Other possible mechanisms include dysfunction of adipose tissue due to dysregulation of adipocytokines such as adiponectin, leptin, and plasminogen activator inhibitor-1 resulting from the enlargement of adipocytes (Shimizu et al., 2011). Thus, the effects of obesity on the promotion of tumor development and the associated cancer cell biology

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have been widely investigated. Nevertheless, the effects of obesity on the early stages of carcinogenesis, and especially the spontaneous occurrence of somatic gene mutations, remain unclear.

Animals carrying reporter genes, such as *gpt* delta transgenic animals, have been utilized to estimate the *in vivo* mutagenicity of environmental chemicals (Nohmi et al., 2000). In addition, these animals have also been used to investigate spontaneous gene mutations affected by specific genetic defects such as p53 or Nrf2 knockout (Aoki et al., 2007; Masumura et al., 2011). In the present study, to determine the effects of obesity on spontaneous gene mutations, we performed reporter gene mutation assays of liver, kidney, and colon, which are organs in which obesity has been associated with cancer development on the basis of epidemiological or animal studies, in high fat diet (HFD)-fed obese mice.

Materials and Methods

Animals

Five-week-old male and female C57BL/6 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 per haploid genome were randomized by body weight into 2 groups. They were housed in polycarbonate cages with hardwood chips for bedding in a conventional animal facility, air-conditioned to 23±2°C and 55±5% humidity, on a 12 hour light-dark cycle. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan).

Animal treatments

Following 1 week of acclimatization, 6-week-old male and female C57BL/6 *gpt* delta mice were fed HFD (Quick fat diet, crude fat; 13.6% [CLEA Japan, Tokyo, Japan]) or standard diet (STD) (CE-2 diet, crude fat; 4.3% [CLEA Japan]) *ad libitum* for 13 or 26 weeks. Body weights were measured once a week. At the end of the experiments, all animals were euthanized under deep anesthesia. Livers, kidneys, and colon mucosa were collected and frozen immediately in liquid nitrogen and stored at -80°C for *in vivo* mutation assays. A portion of each of the harvested livers was fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. At necropsy, blood samples were collected from the abdominal aorta for analysis of serum leptin levels using the mouse leptin Quantikine ELISA Kit (R&D systems, MN).

In vivo mutation assays

6-Thioguanine (6-TG) and Spi⁻ selection were performed as previously reported by Nohmi T. et al. (Nohmi et al., 2000). Briefly, genomic DNA was extracted from liver, kidney, and colon mucosa and lambda EG10 DNA was rescued as phages by *in vitro* packaging. For 6-TG selection, packaged phages were incubated with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, infected cells were also poured onto plates containing chloramphenicol without 6-TG. The

plates were then incubated at 37°C. Positively selected colonies were counted on day 3 and collected on day 4. The *gpt* mutant frequencies (MFs) were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi⁻ selection, packaged phages were incubated with E. coli XL1-Blue MRA for survival titration and E. coli XL1-Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. On the next day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes and the agar plugs suspended in SM buffer. The Spi⁻ phenotype was confirmed by spotting the suspensions on three types of plates on which XL1-Blue MRA, XL1-Blue MRA P2, or WL95 P2 strains were spread with soft agar. Spi⁻ mutants, which produced clear plaques on every plate, were counted.

Statistical analysis

All results were expressed as mean±SD. The data for body weights, *gpt* and Spi⁻ MFs, and serum leptin levels were analyzed by Student's test.

Results

Obesity development

Body weight curves are shown in Figure 1. Final body weights of male mice fed STD or HFD for 13 weeks were 30.6±1.9 g and 36.2±3.2 g, and those of mice fed the diets for 26 weeks were 35.5±3.7 g and 45.2±3.4 g, respectively. Final body weights of female mice fed STD or HFD for 13 weeks were 23.2±1.1 g and 25.7±1.9 g, and those of mice fed the diets for 26 weeks were 26.0±2.0 g and 35.6±5.3 g, respectively. Final body weights of male and female mice fed HFD for 13 or 26 weeks were significantly

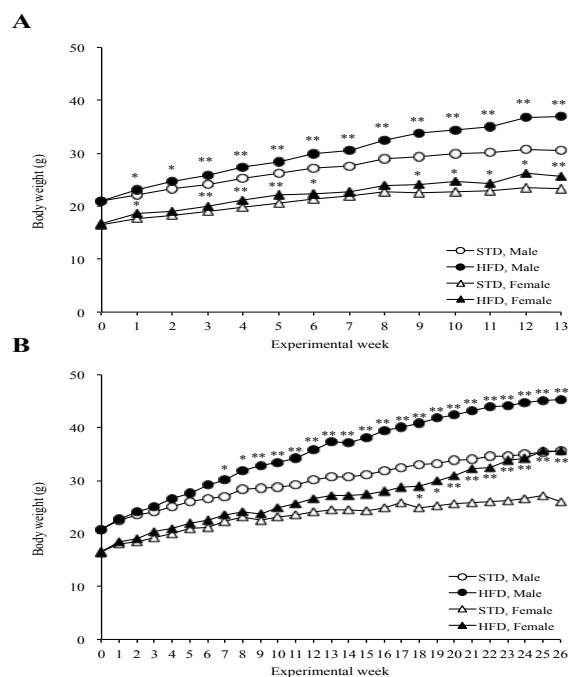


Figure 1. Body Weight Curves for C57BL/6 *gpt* Delta Mice Fed HFD for 13 A) or 26 B) Weeks. *,**, Significantly different from the STD Group at $p < 0.05$ and $p < 0.01$, respectively

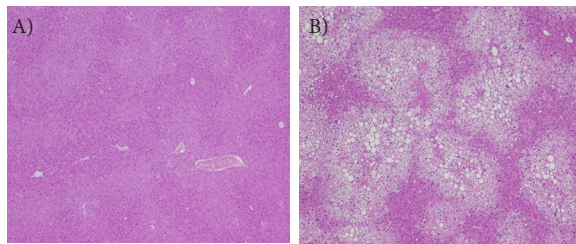


Figure 2. Histopathological Features of the Livers of C57BL/6 *gpt* Delta Mice Fed STD A) or HFD B) for 26 Weeks. HE Stain

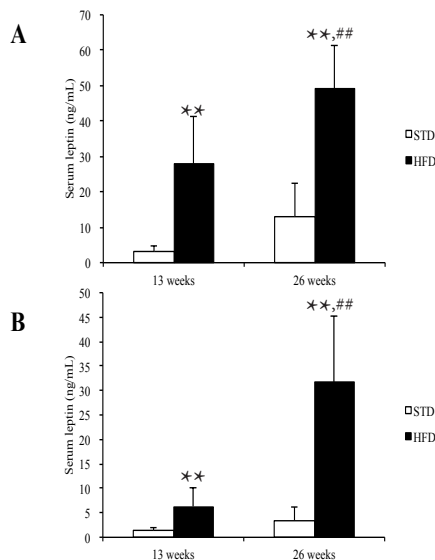


Figure 3. Serum Leptin Concentrations of Male (A) and Female (B) C57BL/6 *gpt* Delta Mice Fed HFD for 13 or 26 weeks. **Significantly different from the STD group at $p < 0.01$. ##, significantly different from the group fed HFD for 13 weeks at $p < 0.01$. Values are means \pm SD

Table 1. *Gpt* Mutant Frequencies in the Liver, Kidney and Colon of Mice Treated with STD or HFD

Organ sex		Mutant frequency ($\times 10^{-5}$) ^a			
		13 weeks		26 weeks	
		STD	HFD	STD	HFD
Liver	Male	0.24 \pm 0.13 ^b	0.22 \pm 0.15	0.73 \pm 0.11	0.61 \pm 0.17
	Female	0.72 \pm 0.24	0.45 \pm 0.19	0.57 \pm 0.19	0.52 \pm 0.21
Kidney	Male	0.27 \pm 0.17	0.36 \pm 0.28	0.67 \pm 0.59	0.49 \pm 0.26
	Female	0.27 \pm 0.12	0.22 \pm 0.11	0.48 \pm 0.32	0.36 \pm 0.21
Colon	Male	0.38 \pm 0.12	0.51 \pm 0.19	0.95 \pm 0.64	0.92 \pm 0.31
	Female	0.50 \pm 0.19	0.63 \pm 0.31	1.10 \pm 0.52	0.80 \pm 0.42

^aMutant frequency were calculated by dividing the number of chloramphenicol and 6-TG resistant colonies by the number of chloramphenicol resistant colonies.
^bMean \pm SD

increased compared with corresponding STD-fed groups. Histopathological examination showed that the livers of mice fed HFD showed substantial steatosis (Figure 2). In addition, the final body weights of male and female mice fed STD or HFD for 26 weeks were also significantly increased compared with those of mice fed STD or HFD for 13 weeks. Serum leptin levels of male and female mice fed HFD for 13 or 26 weeks were significantly increased compared with corresponding controls (Figure 3). In addition, animals that underwent HFD feeding for 26 weeks showed significantly increased serum leptin levels compared with those of animals fed HFD for 13 weeks.

Table 2. *Spi* Mutant Frequencies in the Liver, Kidney and Colon of Mice Treated with STD or HFD

Organ sex		Mutant frequency ($\times 10^{-5}$) ^a			
		13 weeks		26 weeks	
		STD	HFD	STD	HFD
Liver	Male	0.15 \pm 0.09 ^b	0.19 \pm 0.08	0.37 \pm 0.29	0.14 \pm 0.04
	Female	0.16 \pm 0.07	0.12 \pm 0.04	0.50 \pm 0.35	0.23 \pm 0.08
Kidney	Male	0.17 \pm 0.07	0.31 \pm 0.23	0.16 \pm 0.05	0.30 \pm 0.13
	Female	0.16 \pm 0.05	0.19 \pm 0.09	0.29 \pm 0.15	0.19 \pm 0.01
Colon	Male	0.20 \pm 0.16	0.15 \pm 0.04	0.28 \pm 0.11	0.23 \pm 0.09
	Female	0.35 \pm 0.17	0.21 \pm 0.11	0.31 \pm 0.30	0.34 \pm 0.11

^aMutant frequency were calculated by dividing the number of plaques within WL95 (P2) by the number of plaques within XL-1 Blue MRA; ^bMean \pm SD

gpt and *Spi* MFs in liver, kidney, and colon

Data for *gpt* and *Spi* MFs in liver, kidney, and colon from mice fed HFD for 13 or 26 weeks are summarized in Table 1 and 2. There were no significant differences in *gpt* and *Spi* MFs between the STD- and HFD-treated mice for either duration of feeding.

Discussion

In the present study, body weights of mice fed HFD were significantly higher than those of mice fed STD. The degree of body weight gain was increased with HFD feeding duration. These results imply that HFD feeding is responsible for the observed increase in body weight. In addition to the increase in body weights, histopathological analysis revealed that the livers of mice fed HFD exhibited steatosis, indicating that the mice fed HFD under the present experimental conditions became obese due to a metabolic disorder.

In recent years, a strong association between obesity and tumor development has come to be widely accepted. Some studies using rodents have suggested that obesity has the potential to promote tumor development (Hirose et al., 2004; Iwasa et al., 2010; Padidar et al., 2012). Genetically altered obese animal models such as *db/db* mice, which show hepatic steatosis accompanied by obesity (Iwasa et al., 2010; Trak-Smayra et al., 2011), are reported to have a high susceptibility to chemical carcinogenesis of the liver and colon (Hirose et al., 2004; Iwasa et al., 2010).

Meanwhile, since some adipocytokines such as leptin and adiponectin have been reported to affect metabolism, inflammation, angiogenesis, and cell-cycle regulation (Vansaun, 2013), breakdown of their homeostasis due to obesity has been proposed to play an important role in the promotion of tumor development (van Kruijsdijk et al., 2009). Indeed, one of the above-mentioned studies documenting the effects of obesity on the development of colon preneoplastic lesions reported that serum leptin concentrations of mice fed a high fat diet (60% fat by energy) were 4.2 times higher than those of mice fed a low-fat diet (10% fat by energy) (Padidar et al., 2012). Leptin, which is a 16kD bioactive protein mainly secreted from adipocytes, acts as a regulator of energy balance to regulate satiety and modulate glucose and insulin homeostasis (Bjorbaek and Kahn, 2004). Leptin elicits activation of Janus-activated kinase 2 and subsequent phosphorylation of insulin receptor substrates, and subsequently initiates activation of phosphoinositide

3-kinase (PI3K)/Akt pathways through the short-form leptin receptor (Ceddia, 2005). In addition, leptin binding to the long-form leptin receptor activates signal transducer and activator of transcription (STAT) 3 and STAT5 (Ceddia, 2005). Activation of these pathways leads to proliferation of various cells, including neoplastic cells, and promotes growth (van Kruijsdijk et al., 2009). Thus, it has been proposed that hyperproduction of leptin may contribute to tumor development. In the present study, serum leptin concentrations of male mice fed HFD for 13 and 26 weeks were 9.3 and 3.8 times higher, respectively, than those of mice fed STD for the same time periods. Among female mice, serum leptin concentrations in HFD-fed mice were 4.3 and 9.7 times higher than those of mice fed STD for the same time periods. These findings indicate that the degree of obesity under the present experimental conditions was sufficient for investigation of the effects of obesity on spontaneous gene mutations. The tissues examined in the present study, liver, kidney, and colon, were selected because they have been proposed to be associated with obesity-related cancer development on the basis of epidemiological studies (Renehan et al., 2008; World Cancer Research Fund, 2007). Thus, the organs examined in present study were considered to be reasonable target tissues in which to investigate the effects of obesity on the spontaneous occurrence of somatic gene mutations.

To investigate the effects of obesity on spontaneous gene mutations, we performed reporter gene mutation assays of liver, kidney, and colon from mice, and demonstrated that there were no significant differences between the STD- and HFD-fed groups. Our previous data demonstrated that the spontaneous *gpt* MFs in the colon of male C57BL/6 *gpt* delta mice at 11 weeks of age was 0.82 ± 0.43 (Means \pm SD, n=5) (Okamura et al., 2010). The values at 19 and 32 weeks of age in the present study were 0.38 ± 0.12 and 0.95 ± 0.64 , respectively. Therefore, the values of *gpt* MFs in the present study seem to be within the same range of data from the previous study. Thus, the present data showing no significant increase in spontaneous gene mutations in the organs associated with obesity-related cancer even under conditions of severe obesity suggest that HFD treatment and consequent obesity do not affect the frequency of spontaneous somatic gene mutations in spite of the condition being effective on the tumor promotion.

In conclusion, we demonstrated that obesity induced by HFD did not influence the frequency of reporter gene mutations in liver, kidney, or colon from *gpt* delta mice. Therefore, the tumor-promoting effects of obesity may not involve spontaneous gene mutations, suggesting that obesity may contribute only to the promotion of carcinogenesis.

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