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ARTICLE

Leptin increases the expression of tumour promoter genes and decreases the expression of tumour suppressor genes in stomach of female Sprague-Dawley rats

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Abstract: Background: Epidemiologic studies indicate that the incidence of gastric cancer is lower in females than in males due to the protective effects of oestrogen. However, a few reports have found that there was no difference in relative risk of gastric cancer in obese females compared to obese males. Our recent studies have shown that leptin administration promotes tumour growth in the stomach of male Sprague-Dawley rats. However, it is unknown whether leptin can also equally induce gastric tumorigenesis in female rats. This study therefore examined the effects of leptin on tumour development in stomachs of female Sprague-Dawley rats.
Methodology: Female Sprague-Dawley rats aged 6 weeks were divided into 2 groups; leptin group that was given daily intraperitoneal injections of leptin at a dose of 60 µg/kg for 40 weeks (n=8) and a control group that was given daily intraperitoneal injections of an equal volume of normal saline (n=8). Body weights were measured weekly. After 40 weeks, the rats were euthanised and their stomachs collected for histopathological examination and gene analysis. Nine target genes commonly involved in tumorigenesis were selected for RT-PCR, which included *APC*, *ARID1A*, *E-Cadherin*, *FAT4*, *L-Myc*, *PGC*, *PPARG*, *TFF1*, and *TFF2*. Data were analysed using one-way ANOVA.
Results: No macroscopic or microscopic changes were found in the stomach of leptin-treated rats. However, gene analysis revealed significant decrease in tumour suppressor genes like *ARID1A, E-cadherin, APC, TFF1,* and *PPARG*, but not *FAT4*. Tumour promoter genes *L-Myc* and *TFF2* were both significantly upregulated in leptin-treated rats. In situ expression of *PGC*, which is a negative marker for gastric cancer, was found decreased considerably as well. No significant differences in body weight were found between control and leptin-treated rats.
Conclusion: The evident down regulation of some tumour suppressor genes and up-regulation of some tumour promoter genes in leptin-treated rats seem to indicate a tumorigenic potential of leptin in female rats as well, although it may not be as profound as reported in male Sprague-Dawley rats.
Key words: leptin, gastric cancer, tumour suppressor, tumour promoter

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***Abbreviations*:** MNNG, N-Methyl-N'-Nitro-N-Nitrosoguanidine; HE, haematoxylin and eosin; RT-qPCR, Reverse-Transcriptase quantitative PCR; IDT, Integrated DNA Technologies.

***Authors' Contributions*:** The manuscript has been read and approved by all of the authors.

***Competing interests*:** The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

The prevalence of gastric cancer is high in obese individuals who, for some reason, are at an increased risk of developing cancer [1]. Three reasons have been suggested to explain the link between excess adiposity and gastrointestinal cancer risk, including obesity-associated chronic low-grade inflammation, altered insulin signalling, and altered sex hormone metabolism. The role of adipokines in this has not been well investigated. Leptin levels are elevated in overweight and obese individuals. Leptin is an adipocyte-derived hormone that is an essential regulator of appetite and energy expenditure [2]. It suppresses appetite and increases energy expenditure. Leptin levels are elevated in the serum of obese individuals. Leptin has been shown to have cell proliferating properties, particularly of cells from cancers of the breast, lungs, colorectal, uterine, pancreas, and thyroid gland [3–5]. Overexpression of leptin and its receptor is reported in gastric cancer in humans [6]. We recently reported that 40 weeks of daily leptin treatment up-regulated multiple cancer-promoting genes and induced precancerous lesions in the stomach of normal weight male Sprague-Dawley rats [7]. In addition, we also found that leptin enhanced the carcinogenic activity of MNNG in male Sprague-Dawley rats [8]. Now, gastric cancer is reportedly less prevalent in women [9]. The reason/s for this is uncertain but it has been attributed to the protective effect of oestrogen and/or perhaps due to differences in diet and/or occupational exposures [10]. However, recent meta-analyses reported that the relative risk of gastric cancer was higher in obese men, but was not significantly different from that in obese females [11, 12]; implying perhaps that the risk of gastric cancer is higher in obese individuals regardless of the gender. Whether leptin is similarly tumorigenic in normal weight female Sprague-Dawley rats as that reported in the normal weight male Sprague-Dawley rats is unknown. This study therefore investigated the effect of chronic leptin administration on the morphology and histology of the stomach of female rats. In addition, the expressions of nine genes that are commonly involved in tumorigenesis were also determined.

Materials and Methods

Animals and diets

Female Sprague-Dawley rats, aged 6 weeks, were obtained from Laboratory Animal Care Unit (LACU), Faculty of Medicine, UiTM Sungai Buloh, (Selangor, Malaysia). The rats were housed in polypropylene cages with commercial wood chip bedding in the animal room of LACU at an ambient temperature of 22 – 24 °C, relative humidity of 50 – 55 %, and a 12-hour light/dark cycle. Their beddings were changed twice a week. Rats had access *ad libitum* to rat food (Special Feeds Pty Ltd Australia) and tap water. The animal handling and experimental treatment procedures were done in accordance with the International Guidelines for Care and Use of Laboratory Animals (Ref. No. ACUC-8/12) and approved by the Animal Care and Use Committee of the Faculty of Medicine, UiTM (ACUC, Faculty of Medicine, UiTM, Malaysia).

Experimental grouping

Sixteen, Sprague-Dawley, rats weighing 135 – 145 grams were divided equally into control and experimental groups. The experimental group was given intraperitoneal injections of leptin once daily at a dose of 60 µg/kg for 40 weeks. The control group was given daily intraperitoneal injections of an equal volume of normal saline. Body weights were measured weekly. After 40 weeks of treatment, and following an overnight fast, the rats were lightly anesthetized with diethyl ether and then euthanized using a small animal guillotine. Their stomachs were collected and sectioned longitudinally, dividing the lesser and greater curvatures. One half of the stomach from each rat was kept in 10% neutral-buffered formalin for histopathological examination and the other half of the stomach was kept at -80 °C for gene analysis. The dose of leptin of 60 µg/kg used was based on our previous studies investigating the effect of leptin on gastric adenoma in male rats and on studies investigating the impact of leptin on blood pressure, blood pressure and proteinuria during pregnancy, sperm parameters, and precancerous gastric lesions in the rat; where it was found to show significant adverse effects with negligible influence on food intake or body weight [7, 13–17]. The treatment duration of 40 weeks was based on the N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) model of gastric adenocarcinoma [8, 18–21] and on our previous study [6]. Figure 1 depicts the flowchart of the study design.

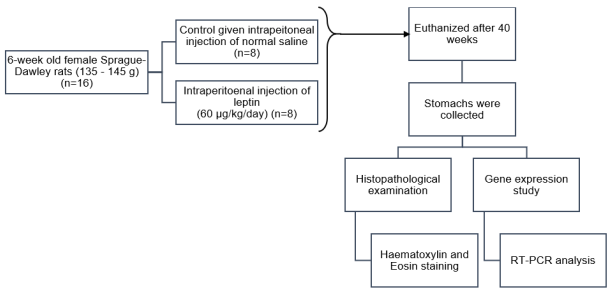

Figure 1. Flowchart of study design.

Histopathological Examination

Macroscopic and microscopic changes of the organs were analysed and confirmed by a senior pathologist (Prof Methil Kannan Kutty). The stomachs were then fixed in 10% neutral buffered formalin, processed, and

embedded in paraffin. Sections were made and stained with haematoxylin and eosin (HE). The stained slides were examined under a digital compound microscope at 400x magnifications.

RT-qPCR Analysis

RNA was extracted from the stomach tissues using innuPREP_RNA Mini Kit (Analytik Jena, Germany) and stored immediately at -20 ºC. The RNA extract was then converted to cDNA using Maxima First Strand cDNA synthesis kit (Thermo Scientific, USA) and incubation was performed using Mastercycler Pro thermal cycler (Eppendorf, Germany). The cDNA was then stored at -20 ºC. Reverse-Transcriptase quantitative PCR (RT-qPCR) was performed to determine the expressions of the nine target genes commonly involved in tumorigenesis using Luminaris Colour HiGreen qPCR Master Mix (2X) (Thermo Scientific, USA). These genes included tumour suppressor genes (*APC, ARID1A, E-Cadherin, FAT4, PPARG, TFF1*), tumour promoter genes (*L-Myc, TFF2*), and negative gastric tumour marker (*PGC*). *GAPDH* and *RPL29* were used as reference genes to normalize the relative gene expressions. The gene expressions were run using PCR thermal cycler CFX 96 (Bio-Rad, USA). Primers were designed using Beacon Designer 8 and synthesized by Integrated DNA Technologies (IDT), USA. Table 1 shows the gene sequences, annealing temperatures, and PCR efficiency values of the target and reference genes.

Statistical analysis

Body weight was analysed using two-way ANOVA, while differences in gene expression were determined using one-way ANOVA. A 'p' value of less than 0.05 was considered statistically significant.

Results

Body weight

The body weight of rats increased significantly over the study period in both the groups. There were, however, no significant differences in body weight between the two groups (Fig. 2).

Macroscopic View of Stomach

No macroscopic changes were evident in the stomach of control or leptin-treated rats. (Fig. 3).

Microscopic View of Stomach

No microscopic changes or differences were found in the stomach of control or leptin-treated rats (Fig. 4).

Gene Expression Analysis using RT-qPCR

Expression of tumour suppressors

Fig. 5A presents the expressions of tumour suppressor (*APC, ARID1A, E-cadherin, FAT4, PPARG,* and *TFF1*) genes in the stomach of rats. Leptin treatment significantly reduced the expression of *APC* (i), *ARID1A* (ii), *E-cadherin* (iii), *PPARG* (v), and *TFF1* (vi) compared to control. However, *FAT4* (iv) expression was significantly up-regulated in leptin-treated rats compared to control.

Table 1. Primer Sequences for RT-qPCR

Genes	Symbols	Ta (°C)	E (%)		Primer Sequences
Adenomatous Polyposis Coli	*APC*	63	93.3	F	5' TGA TGA TGA CGA TGT TGA CCT CTC 3'
				R	5' GCC TTC CTA GCC GAC TGT TG 3'
AT Rich Interactive Domain 1A	*ARID1A*	60	95.8	F	5' AGC CAA GGA GAA CAG AGC AAT 3'
				R	5' AGC GAG ACT GAG CGA CACT 3'
Epithelial Cadherin	*e-Cadherin*	62	101.1	F	5' GCT CGC TGA ACT CCT CTG AG 3'
				R	5' TCG CCG CCA CCA TAC ATA TC 3'
FAT Atypical Cadherin 4	*FAT4*	57	99.2	F	5' TCA CAT CAG GAG TCA CAA GTCT 3'
				R	5' CCA TCG CTG CTA ACC ACA 3'
Peroxisome Proliferator-activated	*PPARG*	60	101	F	5' GCA GGA GCA GAG CAA AGA G 3'
receptor gamma				R	5' GGA CAC CAT ACT TGA GCA GAG 3'
Trefoil factor 1	*TFF1*	58	96.2	F	5' AGA ATA AAT TGT GGC TTC CC 3'
				R	5' TTC TCT CGG ATG GAC CTT AG 3'
Myelo-cytomatosis	*L-Myc*	60	100.2	F	5' AGA GGA GGA GGA GGA GGA AG 3'
				R	5' GGT GGC AGG ACT GAG GAG 3'
Trefoil factor 2	*TFF2*	60	107.6	F	5' ACC AAG CGT CGG AAC AAT G 3'
				R	5' AAG AAA CAC CAG GGC ACT TC 3'
Pepsinogen C	*PGC*	55	94.8	F	5' TCC ACT TAC TAC ACC GAA GG 3'
				R	5' TAC CAG GCT CAT TCT CAC TC 3'
Glyceraldehyde 3-phosphate	*GAPDH*	63	103.1	F	5' CAA CTC CCT CAA GAT TGT CAG CAA 3'
dehydrogenase				R	5' GGC ATG GAC TGT GGT CAT GA 3"
Ribosomal Protein L29	*RPL29*	60	96.4	F	5' CAA GGG TCG TAG GCT CTG 3'
				R	5' AGA TAG TGT GTA GGT GGG TGT 3'

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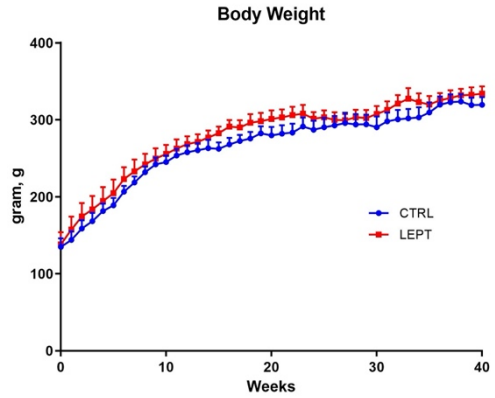

Figure 2. Body weight of rats. No significant differences in body weight between control and leptin-treated rats.

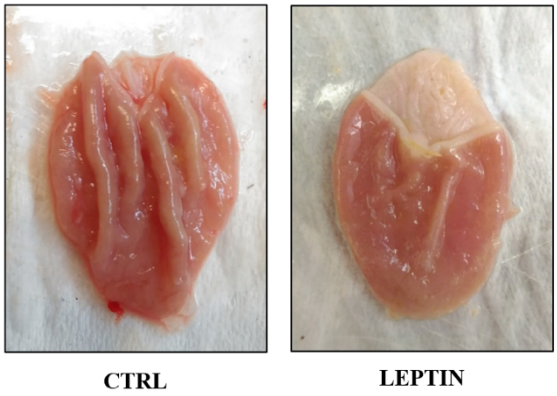

Figure 3. Macroscopic view of stomach of rats. No tumours were found in control and leptin-treated stomachs.

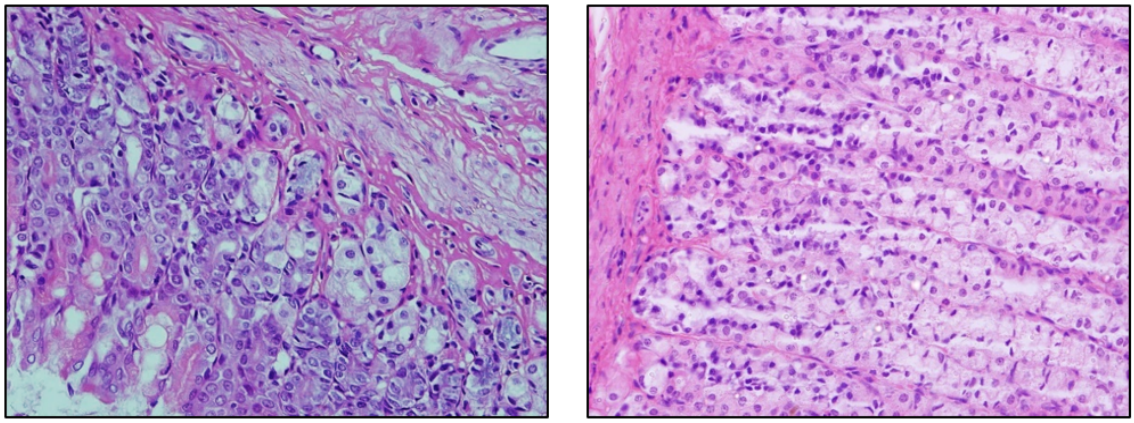

Figure 4. Microscopic view of stomach of rats. No cellular morphological changes were found in control and leptin-treated stomachs.

Expression of tumour promoters

Fig. 5B shows the expression of tumour promoter *L-Myc* and *TFF2* genes in the stomach of rats. Leptin treatment significantly increased the expression of *L-Myc* (i) and *TFF2* (ii) compared to control.

Expression of negative gastric tumour marker

Fig. 5C shows the expression of *PGC* gene (negative gastric tumour marker) in the stomachs of control and leptin-treated rats. Leptin treatment significantly reduced the expression of *PGC* compared to control.

Discussion

The major findings of this study include (i) the absence of either macroscopic (Fig. 3) or microscopic (Fig. 4) changes in the stomach of leptin-treated and control rats; (ii) significant down-regulation in the expressions of tumour suppressor genes like *APC, ARID1A, E-cadherin, PPARG,* and *TFF1* in stomachs of leptin-treated rats, except for *FAT4* gene, which was significantly up-regulated (Fig. 5A); (iii) significant up-regulation in the expression of tumour promoter *L-Myc* and *TFF2* genes in the stomach of rats treated with leptin (Fig. 5B); and (iv) down-regulation in the expression of negative gastric tumour marker *PGC* gene in leptin-treated rats (Fig. 5C).

Although leptin treated rats did not develop any obvious signs of gastric tumours as we had reported earlier in the male rats [7], the finding of down-regulation of tumour suppressor genes and up-regulation of some of the tumour promoter genes in these female Sprague-Dawley, nevertheless, suggest of a potential role of leptin in gastric tumorigenesis in female Sprague-Dawley rats as well. The findings that leptin might have a tumorigenic effect are also in agreement with our previous study in male Sprague-Dawley rats [7] and also that of other studies [22–24], although the effects were not as profound as those in male Sprague-Dawley rats. The precise reason for the lack of any macroscopic or microscopic

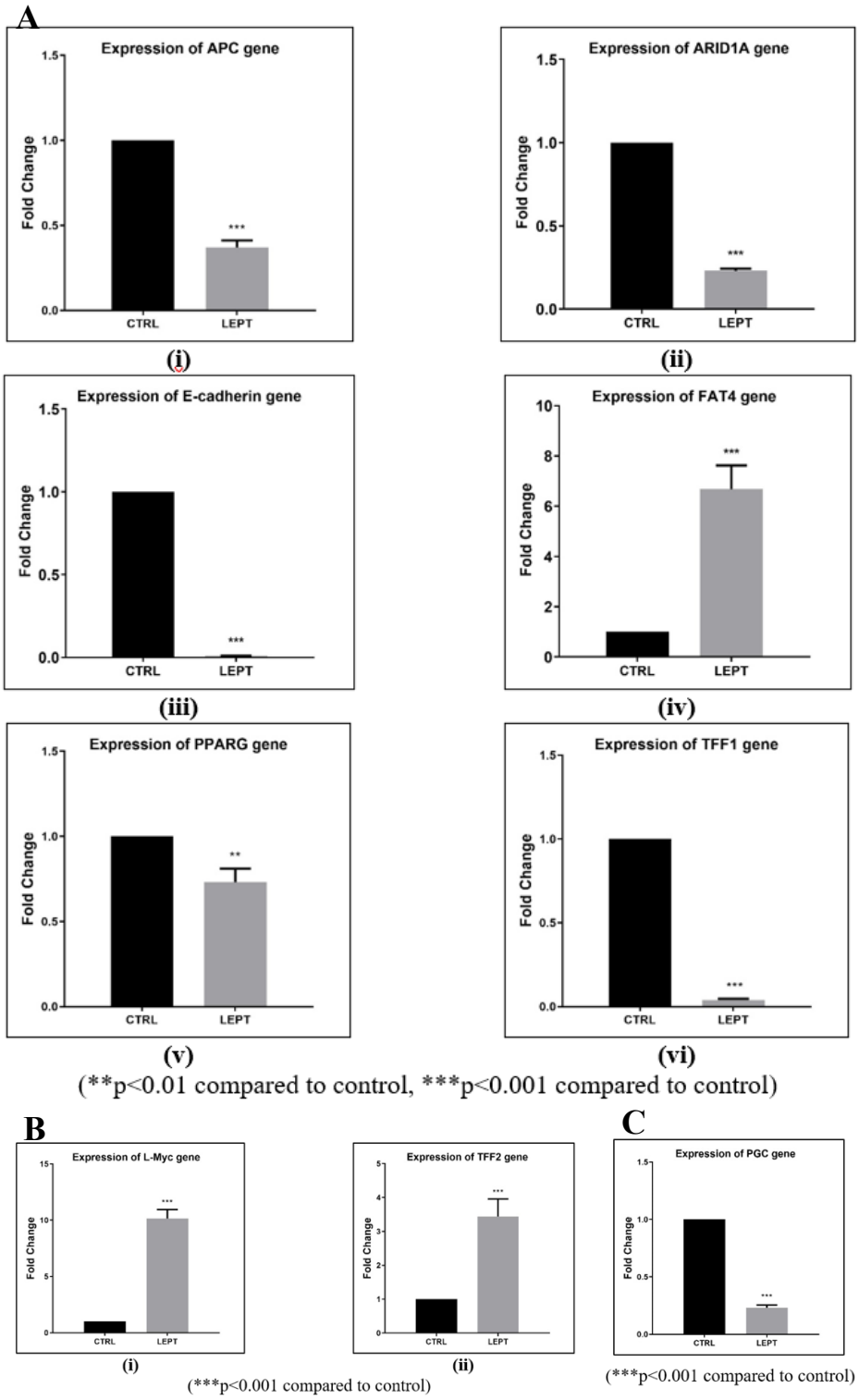

Figure 5. A. Expressions of APC (i), ARID1A (ii), E-cadherin (iii), FAT4 (iv), PPARG (v), and TFF1 (vi) gene. The expression of APC, ARID1A, E-cadherin, PPARG, and TFF1 was significantly lower in leptin-treated stomachs. However, FAT4 expression was significantly higher in leptin-treated stomachs. B. Expressions of L-Myc (i) and TFF2 (ii) gene. The expression of L-Myc and TFF2 was significantly higher in leptin-treated stomachs. C. Expressions of PGC gene. The expression of PGC was significantly lower in leptin-treated stomachs.

changes in the stomachs of these leptin-treated female rats remains unclear. Male Sprague-Dawley rats that were injected with leptin for 40 weeks had obvious macroscopic and microscopic changes [7]. This finding might perhaps have some relevance to the lower prevalence of gastric carcinoma in women, which has

TMR
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been attributed to higher oestrogens that are normally present in women during the reproductive years [11, 12]. The overall risk of developing gastric cancer up to the age of 75 in males is 1.87% whereas in females it is 0.79% [25]. It is unclear whether a longer duration of treatment or perhaps even higher doses of leptin are required to induce microscopic and macroscopic changes in the stomachs of female Sprague-Dawley rats. It is, however, also interesting to note that although, in general, the prevalence of gastric cancer is lower in females, there is however no difference in its prevalence between obese men and women [11, 12].

Oestrogen is known to protect against malignancies as gastric cancer risk is reportedly lowered by delayed menopause, in those on hormonal replacement therapy, and in men treated with oestrogen for prostate cancer [26]. However, metabolically abnormal obese individuals had an elevated risk of gastric cancer [27], and visceral adiposity was associated with increased risk of colorectal cancer in both men and women [28]. Leptin levels are significantly higher in obese individuals, and it is possible that the increased risk of cancer in both obese men and women could be due to the higher leptin levels. This study reports the probable association of leptin with tumour suppressors *APC, ARID1A, E-cadherin, PPARG,* and *TFF1*, tumour promoters *L-Myc* and *TFF2*, and negative gastric tumour marker *PGC* in the stomach of female rats. To the best of our knowledge, this is the first study to have done so.

In this study, leptin significantly reduced the expression of tumour suppressors *APC, ARID1A, E-cadherin, PPARG,* and *TFF1* genes in leptin-treated stomachs (Fig. 5A). Loss of *APC* was reported in early stage gastric carcinoma [29], and recently, *APC* missense mutations were found in gastric tumours and was associated with poor survival [30]. Loss of *ARID1A* expression is associated with poor overall survival in early-stage undifferentiated gastric cancer [31]. Low *E-cadherin* expression found in gastric cancer were significantly correlated with tumour stage and metastasis [32]. *PPARG* agonists inhibited proliferation of gastric cancer cell lines possibly through induction of apoptosis [33]. *TFF1* plays a role in providing gastrointestinal mucus barrier and its expression was significantly reduced in gastric carcinoma [34]. Leptin, in addition to enhancing the activity of known tumour promoters [4], may also decrease these tumour suppressors to induce carcinogenesis in the stomach of female rats. However, *FAT4* gene, which is a known tumour suppressor, was found up-regulated in leptin-treated rats (Fig. 5A). Silencing of *FAT4* gene promoted epithelial-to-mesenchymal transition and invasion of gastric cancer cells [35]. Hence, *FAT4* plays an important role in suppressing the growth and metastasis of gastric cancer cells [35]. This shows that leptin may not down-regulate all tumour suppressors, and up-regulation of *FAT4* is perhaps to suppress gastric cancer growth.

Leptin significantly increased the regulation of tumour promoter genes *L-Myc* and *TFF2* (Fig. 5B). *L-Myc* is a member of the *Myc* family gene and is a proto-oncogenic transcription factor that was found commonly overexpressed in gastric cancer [36]. Overexpression of *TFF2* may promote gastric tumour progression by increasing tumour vascularity and correlated with poor prognosis [37]. Leptin augmented the expression of both these tumour promoters, which suggests that leptin is promoting carcinogenesis in the stomach of female rats.

The negative gastric tumour marker *PGC* gene was found significantly down-regulated in the stomach of leptin-treated rats. *PGC* is synthesized by chief cells of normal gastric mucosa, and its expression is reportedly decreased considerably from gastritis to gastric cancer [38]. Although gastric cancer was not found in this study, the reduced expression of *PGC* suggests that leptin is gradually changing the morphology of the normal gastric mucosa. Hence, perhaps with a longer duration of leptin treatment, these phenotypic changes could become evident.

The body weight of female Sprague-Dawley rats increased significantly throughout the study period, and there were no significant changes in body weight evident between the two groups (Fig. 2).

In conclusion, this study has demonstrated that despite no visible changes observed in gross and histopathological features of leptin-treated stomachs in female rats, gene expression analysis clearly showed that leptin significantly altered the genes that favour tumorigenesis compared to control. This clearly indicates the cancer-promoting effect of leptin in the stomachs of female rats, albeit not as profound as that observed in male Sprague-Dawley rats.

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