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Effects of two types of energy restriction on methylation levels of adiponectin receptor 1 and leptin receptor overlapping transcript in a mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model

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Abstract

The role of adiponectin and leptin signalling pathways has been suggested to play important roles in the protective effects of energy restriction (ER) on mammary tumour (MT) development. To study the effects of ER on the methylation levels in adiponectin receptor 1 (*AdipoR1*) and leptin receptor overlapping transcript (*Leprot*) genes using the pyrosequencing method in mammary fat pad tissue, mouse mammary tumour virus-transforming growth factor-α (MMTV-TGF-α) female mice were randomly assigned to *ad libitum* (AL), chronic ER (CER, 15 % ER) or intermittent ER (3 weeks AL and 1 week 60 % ER in cyclic periods) groups at 10 weeks of age until 82 weeks of age. The methylation levels of *AdipoR1* in the CER group were higher than those in the AL group at week 49/50 ($P < 0{\cdot}05$), while the levels of methylation for *AdipoR1* and *Leprot* genes were similar among the other groups. Also, the methylation levels at CpG2 and CpG3 regions of the promoter region of the *AdipoR1* gene in the CER group were three times higher ($P < 0{\cdot}05$), while CpG1 island of *Leprot* methylation was significantly lower compared with the other groups ($P < 0{\cdot}05$). Adiponectin and leptin gene expression levels were consistent with the methylation levels. We also observed a change with ageing in methylation levels of these genes. These results indicate that different types of ER modify methylation levels of *AdipoR1* and *Leprot* in different ways and CER had a more significant effect on methylation levels of both genes. Epigenetic regulation of these genes may play important roles in the preventive effects of ER against MT development and ageing processes.

Key words: Intermittent energy restriction: Methylation: Pyrosequencing: *AdipoR1*: *Leprot*: Ageing: Mouse mammary tumour virus-transforming growth factor-α mice: Epigenetics

Breast cancer (BC) is one of the most widespread diseases among women. In 2018, World Cancer Research Fund International reported that 2·09 million women were diagnosed with BC and 626 679 deaths were related to BC invasion[1]. On the other hand, studies have reported that energy restriction (ER) prolongs life span and has protective effects on a variety of diseases including BC[2–4]. Two common ER methods were applied in previous rodent studies: chronic energy restriction (CER) and intermittent energy restriction (IER), both of which, and especially IER, were shown to prevent mammary tumour (MT) development.

Adipokines, particularly adiponectin and leptin, have been associated with obesity and in BC development in both *in vivo* and *in vitro* studies[5,6]. Adiponectin, a 30 kDa protein, is encoded by the *ADIPOQ* gene and is abundantly expressed in adipose tissue[7–10] that has two receptors, adiponectin receptor 1

Abbreviations: *AdipoR1*, adiponectin receptor 1; AL, *ad libitum*; BC, breast cancer; BW, body weight; CER, chronic energy restriction; ER, energy restriction; IER, intermittent energy restriction; IER-R, IER-restriction; IER-RF, IER-refeed; *Leprot*, leptin receptor overlapping transcript; MMTV-TGF-α, mouse mammary tumour virus-transforming growth factor-α; MT, mammary tumour.

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(*AdipoR1*) and adiponectin receptor 2[11–13]. *AdipoR1* has higher affinity than adiponectin receptor 2; thus, it has more definite roles in BC development[14–16]. It has been demonstrated that adiponectin plays significant roles specifically in cancer cell growth, migration, insulin sensitivity, lipid metabolism and glucose regulation[16–19]. In this manner, previous studies reported a growth inhibitory effect of adiponectin on BC development and this effect was suggested to be mediated by *AdipoR1*[20–25]. In addition, BC risk in women with high adiponectin levels was reported to be 65 % lower compared with risk rates of the control group[26,27]. On the other hand, there are studies that reported correlation between lower serum adiponectin levels and lower MT incidence in rodents[3] or no significant difference of serum adiponectin levels between MT-developed and MT-free groups[28,29].

Leptin is another adipokine encoded by *LEPTIN (LEP)* gene and produced primarily by adipose tissue[30]. It acts as a neurohormone that regulates energy balance and food intake[31]. The physiological actions of leptin are controlled by leptin receptor (ObR), a single membrane-spanning receptor homologous to members of the class I cytokine receptor[32–34]. Many studies have reported promoting effects of leptin on BC development by using cell culture and animal experiments[3,32,35,36]. For instance, incubation of T47D cells with leptin increased proliferation of cancer cells by 60–138 %[31,37,38]. In addition, a MT mouse model study demonstrated that animals with lower serum leptin levels had lower MT occurrence rate compared with the ones with high MT occurrence[3]. Likewise, BC patients had significantly higher serum leptin levels compared with the levels of healthy patients[39,40]. Roles of leptin receptor overlapping transcript (*Leprot*) gene that regulates leptin receptor activation[41,42] have been noted on high-fat diet-induced obesity, deleterious phenotypes of metabolic traits, such as higher fasting glucose and total cholesterol levels, and type 2 diabetes mellitus[42,43]. Therefore, it is important to examine the roles of adiponectin, leptin and their receptor signalling pathways in detail in order to have better understanding of the nutritional effects on cancer development[31]. In this context, investigation of the epigenetic mechanisms on the regulation of these genes may be an important approach.

The main aim of the present study was to understand the role of long-term application of two different types of ER on the methylation levels of *AdipoR1* and *Leprot* genes which are involved in both BC and obesity development using MT incidence rate of mouse mammary tumour virus-transforming growth factor-α (MMTV-TGF-α) female mice. In order to further study the underlying mechanism of this phenomenon, methylation levels of promoter regions of both genes were studied using a pyrosequencing strategy. Our hypothesis was that ER modulates adiponectin and leptin levels by regulating *AdipoR1* and *Leprot* methylation levels at specific promoter regions. Thus, these epigenetic changes may play important roles in the protective effects of ER, especially by CER.

Materials and methods

Animals and study design

MMTV-TGF-α (C57/BL6) female mice, developed in the laboratory of Dr Robert J. Coffey, were used in the present study. Originally, MMTV-TGF-α gene positive male mice were provided by Dr Margot Cleary, Hormel Institute, University of Minnesota, to establish a breeding colony at Yeditepe University Animal Facility (YUDETAM). MMTV-TGF-α mice develop MT in the second-half of their lives and have several similarities with human BC. These mice over-express human TGF-α which is a part of the epidermal growth factor receptor/ErbB cascade that plays an important role in BC development. Donated MMTV-TGF-α positive male mice were bred with TGF-α negative female mice (C57BL/6) to obtain heterozygote MMTV-TGF-α female offspring. The presence of TGF-α gene insert was determined by PCR using forward primer 5′-GATCCAGTGTGACCTAGAGAAGAAAT-′3 and reverse primer 5′-GATCTTTTCTATGGAATAAGGAATGGA-′3 and run in 1 % agarose gel electrophoresis. Mice were allowed free access to tap water and were housed individually under standard conditions at a temperature of 21–24°C and 12 h light–12 h dark cycle. Animals were observed for any health problems on a daily basis. All procedures were performed under the guidelines and with the approval of Yeditepe University Animal Care and Use Committee (file approval no. 390, 27 March 2014).

At 10 weeks, MMTV-TGF-α (C57BL6) female mice were randomly enrolled into three different dietary groups: *ad libitum* (AL), CER or IER. All mice were fed with an Altromin TPF1414 diet that was purchased from Kobay AS. Mice in the AL group had free access to food throughout the study. Mice assigned to the CER group were provided 85 % of the daily food consumption of the age-matched AL group, resulting in a 15 % energy reduction compared with the AL group. Mice assigned to the IER group were given 40 % of the food consumed by the AL mice at the same age for 1 week (IER-restriction, IER-R) and then were fed AL for the following 3 weeks (IER-refeed, IER-RF) which also resulted in an overall energy reduction of 15 % compared with AL mice. This cyclical protocol was applied to IER mice until they were killed at designated time points.

All mice were fed daily at 09.00 hours and at the same time, food consumption was measured. Body weights (BW) were measured every Monday at 09.00 hours. The health of the animals was checked weekly by a veterinarian. At designated ages, mice were fasted overnight and then blood samples were collected by retro-orbital bleeding at 09.00 hours. After killing, tissue samples were removed and a piece of each tissue sample was placed in 10 % neutral-buffered formalin to be sent to the Department of Pathology, Yeditepe University School of Medicine for histopathological analyses to determine malignancy and/or disease status in a blind fashion. The designated ages were 10 (baseline), 17 and 18; 49 and 50; and 81 and 82 weeks of age. IER mice were further divided into two groups. Mice from which blood samples were collected at the end of 3 weeks of AL feeding (weeks 17, 49 and 81) were referred as IER-RF. Mice from which blood samples were collected at the end of the 1 week of ER period (weeks 18, 50 and 82) were referred as IER-R. Previous studies conducted by us at Yeditepe University indicated that there were no differences for several parameters including BW of AL and CER group for the 1-week differences. Therefore, the data were combined for 17 and 18; 49 and 50; 81 and 82 at each time point for AL and CER groups. The designated time points were selected as

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referring to the early age (week 10), middle age when MT occurrence generally begins (week 49/50) and old age (week 81/82).

Methylation measurements by pyrosequencing

DNA isolation. Genomic DNA from randomly selected mammary fat pad tissue samples was isolated from each group using Macherey-Nagel DNA Isolation Tissue Kits (Macherey-Nagel). Briefly, 25 mg of mouse mammary fat pad tissues was cut into small pieces and mixed with Buffer T1 and Proteinase K enzyme solution. Then, tissues with lysis solution were incubated on a shaker at 56°C overnight. After the incubation, samples were vortexed and then incubated at 70°C for 10 min. Samples were then added to the column, centrifuged and washed to obtain pure DNA in elution buffer. DNA concentration and quality were assessed using a Nanodrop (Spectrophotometer, NanoPhotometer, Implen) and also by 1 % agarose gel electrophoresis.

Bisulphite conversion and sequencing. In order to study the methylation levels, bisulphite conversion was performed that involves converting cytosine to uracil while leaving 5-methylcytosine intact. Bisulphite conversion was performed using the EpiTect Bisulfite kit (EpiTect Bisulfite kit, QIAGEN GmbH) according to the manufacturer's protocol. Conversion was performed in a thermal cycler using the following conditions: 5 min at 95°C, 25 min at 60°C, 5 min at 95°C, 85 min at 60°C, 5 min at 95°C, 175 min at 60°C and a final step at 20°C. For purification, samples were mixed with 560 µl BL buffer containing 10 µg/ml carrier RNA and loaded into EpiTech spin columns and eluted in 15 µl of buffer. Then, bisulphate-converted DNA samples were amplified by PCR with biotinylated *AdipoR1* and *Leprot* PCR primers that were commercially obtained (QIAGEN GmbH) and mixed with a special master mix containing HotStar Taq DNA Polymerase (QIAGEN GmbH), CoralLoad and Q-solution. Amplification conditions were 15 min at 95°C, 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, for 45 cycles and 10 min at 72°C. Pyrosequencing analysis was conducted on 10 µl of PCR products following the manufacturer's protocol (QIAGEN GmbH). The methylation assays for *AdipoR1* and *Leprot* contained 3 and 7 promoter region CpG, respectively (See online Supplementary Table S1).

Quantitative real-time PCR analysis to measure adiponectin receptor 1 and leptin receptor overlapping transcript mRNA expression levels in mammary fat pad samples

To correlate the methylation levels with the active gene expression, mRNA levels were checked by quantitative real-time PCR. Total RNA was isolated from frozen mouse mammary fat pad tissues, which was stored at −80°C, using a Direct-zol RNA MiniPrep Kit (Zymo Research), and then RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The integrity of total RNA was checked by 1 % agarose gel electrophoresis. cDNA conversion was performed from 200 ng RNA using the iScript cDNA Synthesis kit (Biorad). The PCR primers were designed using nucleotide sequences for mouse *AdipoR1*, *Leprot* and *β*-Actin. The primer sequences were as follows: forward primer 5′-ACGTTGGAGAGTCATCCCGTAT-3′, reverse primer 5′-CTCTGTGTGGATGCGGAAGAT-3′ for *AdipoR1*; forward primer 5′-ATGGAGGGGAATACAGCCC-3′, reverse primer 5′-TTCTTTGCAGCTCCTTCGTT-3′ for *β*-actin gene and forward primer 5′-ACTATGGCGTTTACTGGCCC-3′, reverse primer 5′-AATACGCCAGTTCCCGACAG-3′ for *Leprot*. Quantitative real-time PCR was performed using iTaq Universal SYBR Green Supermix (Biorad) following the manufacturer's protocol using the BioRad CFX96 Touch Real-Time PCR Detection System. Distilled water was used as the negative control, and *β*-Actin values from the same samples were used for normalisation.

Statistical analysis

Methylation analyses were conducted using Prism Software version 3.02, and mRNA expression analyses were conducted using Prism Software Version 7. Results are presented as mean values and standard deviations. Comparisons among groups were made by ANOVA followed by post-Tukey's test to determine whether the differences between specific groups were significant. Since there was no significant difference between two consecutive time points at weeks 17 and 18, 49 and 50, 81 and 82 for AL and CER mice, data from each of these two time points were combined. Statistical significance was set at $P \leq 0{\cdot}05$. '*n*' refers to the number of individual mice in each group, and *n* is between 3 and 4 unless indicated otherwise. Sample sizes were determined by *a priori* computation using effect size f as 1·25 (high effect size), power as 0·8 and α as 0·05 (two-sided significance level) using G*Power 3.1 software.

Results

Effects of different types of energy restriction on methylation of the promoter region of adiponectin receptor 1 gene

AdipoR1 methylation levels of AL, CER, IER-RF and IER-R groups at week 49/50 were 2·75, 2·33, 2·50 and 2.92 %, respectively (Fig. 1(a); $F = 1{\cdot}8$; $P > 0{\cdot}05$). In contrast, *AdipoR1* methylation level was 3·7 times higher in CER mice compared with AL mice at week 81/82 ($F = 4{\cdot}6$; $P < 0{\cdot}05$, Fig. 1(b)). *AdipoR1* methylation levels of AL, CER, IER-RF and IER-R were 3·67, 13·83, 6·08 and 5·83 %, respectively.

The *AdipoR1* methylation assay included three CpG sites. Analysis of individual sites shows that at week 81/82, methylation levels of CpG2 and CpG3 differed significantly among the groups (Table 1; $F = 3{\cdot}8$ for CpG2 and $F = 4{\cdot}3$ for CpG3; $P < 0{\cdot}05$ for both CpG2 and CpG3). In the CER group, methylation levels of CpG2 and CpG3 were approximately three times higher than that in the other groups (Table 1), while methylation level of CpG1 in the CER group was five and two times higher than the AL and IER groups, respectively. There was no significant difference among the groups for the methylation levels of any of the CpG in the *AdipoR1* assay at week 49/50 ($F = 1{\cdot}2$, $F = 0{\cdot}2$, $F = 2{\cdot}3$ for CpG1, CpG2 and CpG3, respectively; $P > 0{\cdot}05$).

Changes in adiponectin receptor 1 promoter region methylation levels with ageing

No significant change was observed in AL and IER-R groups for *AdipoR1* methylation levels with ageing (Fig. 2(a) and (d);

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Table 1. Methylation levels of individual CpG islands of adiponectin receptor 1 (*AdipoR1*) gene in mammary fat pad samples of mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model

AdipoR1	CpG1	CpG2	CpG3	Mean of CpG
Week 10	5·50	4·25	5·00	4·92
Week 49/50				
AL	2·50	2·25	3·50	2·75
CER	2·00	2·33	2·67	2·33
IER-RF	1·75	2·25	3·50	2·50
IER-R	2·50	2·50	3·75	2·92
Week 81/82				
AL	3·33	3·00[a]	4·67[a]	3·67
CER	15·00*	13·75[b]*	12·75[b]*	13·83
IER-RF	7·00*	5·25[a,b]	6·00[a,b]	6·08
IER-R	7·00	5·00[a,b]	5·50[a,b]	5·83

AL, *ad libitum*; CER, chronic energy restriction; IER-RF, intermittent energy restriction-refed; IER-R, intermittent energy restriction-restricted.
[a,b] Values with unlike superscript letters were significantly different among energy-restricted groups at the same time point.
* Statistical significant changes in the CER and IER-RF groups by ageing.

$F = 2·1$; $P > 0·05$). However, methylation levels were increased at week 81/82 compared with earlier ages in CER and IER-RF groups (Fig. 2(b); $F = 7·4$; $P < 0·05$ and Fig. 2(c); $F = 4·3$; $P < 0·05$). In detail, methylation level of *AdipoR1* was significantly higher at week 81/82 compared with either that at weeks 10 or 49/50 in the CER group. Methylation level of *AdipoR1* was significantly higher at week 81/82 than at week 49/50 in the IER-RF group. Analysis of the three individual CpG sites in the *AdipoR1* assay showed that methylation levels of all three CpG sites were significantly increased in CER by week 81/82, while it was only increased in CpG1 for the IER-RF group (Table 1).

Effects of different types of energy restriction on methylation of promoter region of leptin receptor overlapping transcript gene

Leprot methylation levels of AL, CER, IER-RF and IER-R mice at week 49/50 were 2·32, 2·36, 1·93 and 2·25 %, respectively (Fig. 3(a); $F = 0·2$; $P > 0·05$). Although methylation level in the CER group was approximately 2-fold lower compared with the other groups, there was no significant effect among different dietary groups on methylation of *Leprot* gene at week 81/82 (Fig. 3(b); $F = 1·8$; $P > 0·05$). Methylation levels of *Leprot* gene were 4·07, 1·71, 5·10 and 3·96 % for AL, CER, IER-RF and IER-R groups, respectively. The *Leprot* methylation assay included seven CpG sites. Methylation level of CpG1 was approximately three- to five-fold lower compared with the other groups at week 81/82 (Table 2; $P < 0·05$).

Changes in leptin receptor overlapping transcript gene promoter region methylation levels with ageing

There was no significant difference among the dietary groups in terms of changes in the *Leprot* gene promoter region methylation levels with ageing (online Supplementary Fig. S1A; $F = 1·7$; $P > 0·05$). However, analysis of the individual CpG sites revealed a significant increase in methylation levels of CpG1 and CpG2 sites at week 81/82 compared with the week 49/50 in IER-RF group (Table 2).

Effects of different types of energy restriction on mRNA expression levels of adiponectin receptor 1 and leptin receptor overlapping transcript genes in mammary fat pad tissue

Levels of *AdipoR1* gene mRNA expression at weeks 49/50 and 81/82 were similar among all dietary groups (Fig. 4(a) and (b), $P > 0·05$). Also, ageing did not have any significant effect on mRNA expression levels of *AdipoR1* gene in any of the dietary groups (Fig. 4(a) and (b); $P > 0·05$).

Leprot mRNA expresssion was significantly higher in the IER-RF group compared with both the IER-R and CER groups at week 49/50 (Fig. 4(c); $F = 4·1$; $P < 0·05$). On the other hand, there was no significant difference among dietary groups for mRNA expression levels of *Leprot* gene at week 81/82 (Fig. 4(d), $P > 0·05$). *Leprot* expression level was significantly increased at week 49/50 compared with week 10 in the IER-RF group ($P < 0·05$), while *Leprot* gene expression level did not differ by ageing in the other dietary groups ($P > 0·05$).

Discussion

In the present study, the effects of two different types of ER (CER and IER) on the methylation levels of *AdipoR1* and *Leprot* genes which play important roles in adipocytokine signalling pathways

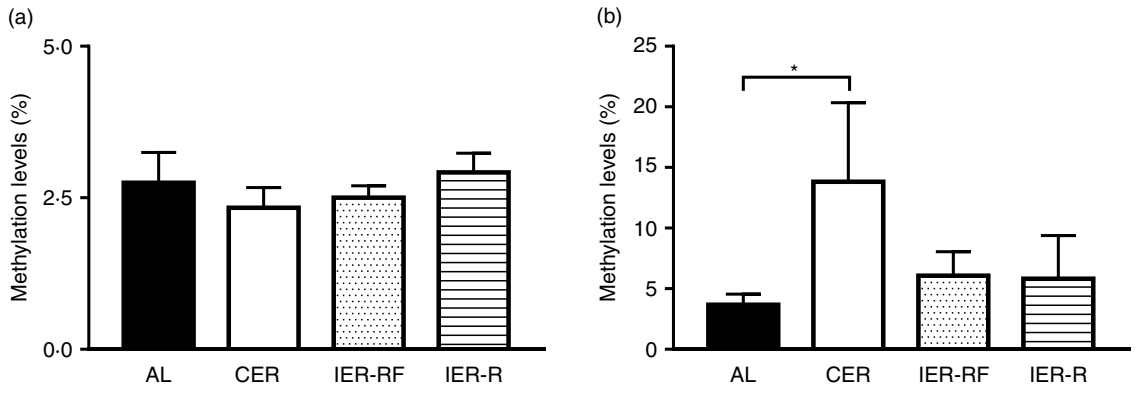

Fig. 1. Effects of different types of energy restriction on adiponectin receptor 1 (*AdipoR1*) methylation levels in mammary fat pad samples of mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model at week 49/50 (a) and at week 81/82 (b). AL, *ad libitum*; CER, chronic energy restriction; IER-RF, intermittent energy restriction-refed; IER-R, intermittent energy restriction-restricted. Values are means, with standard deviations represented by vertical bars. * $P < 0·05$.

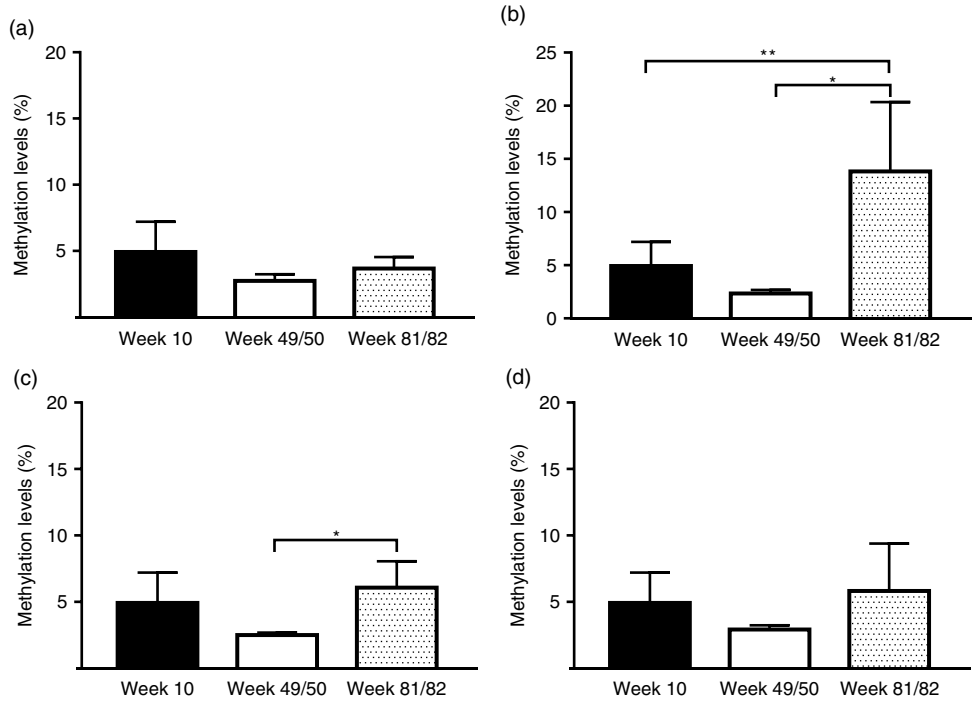

Fig. 2. Effects of ageing on adiponectin receptor 1 (*AdipoR1*) gene methylation levels in mammary fat pad samples of mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model in *ad libitum* (a), chronic energy restriction (b), intermittent energy restriction-refed (c) and intermittent energy restriction-restricted (d) groups. '*n* value' represents samples taken from different animals: *n* 3–4. Values are means, with standard deviations represented by vertical bars. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

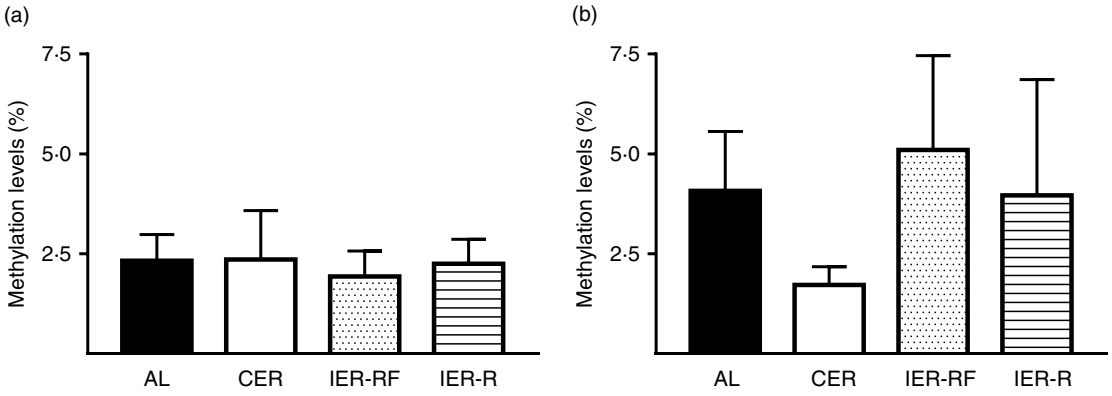

Fig. 3. Effects of different types of energy restriction on leptin receptor overlapping transcript (*Leprot*) methylation levels in mammary fat pad samples of mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model at week 49/50 (a) and at week 81/82 (b). AL, *ad libitum*; CER, chronic energy restriction; IER-RF, intermittent energy restriction-refed; IER-R, intermittent energy restriction-restricted. Values are means, with standard deviations represented by vertical bars.

were studied using mammary tissue obtained from MMTV-TGF-α female mice. Similar to the previous studies, CER protocol had an impact on mice resulting in significantly lower BW gain compared with the AL or IER group. As predicted, mice in IER group lost a significant amount of BW during restriction periods and gained their BW back during the refeeding periods over the course of the study (unpublished results, article under review)[44–48]. Our results indicated that the CER group was more protected against MT development compared with AL and IER groups, that is, since CER mice had lower MT incidence compared with either AL or IER groups, while there was no difference between AL and IER groups (unpublished results, article under review).

The main objective of the present study was to analyse the effects of different types of ER on the methylation levels and expression of *AdipoR1* and *Leprot* genes at different time points throughout the life cycle using a MMTV-TGF-α BC mouse model, from week 10 until week 82 of mouse age. *AdipoR1* and *Leprot* genes were selected due to their significant roles in adiponectin and leptin signalling pathways, which have been suggested to be involved in the processes of MT development and obesity[3,11,46]. Therefore, it is worth studying the epigenetic regulation of these

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Table 2. Methylation levels of individual CpG islands of the leptin receptor overlapping transcript *(Leprot)* gene in mammary fat pad samples of mouse mammary tumour virus-transforming growth factor-α breast cancer mouse model

Leprot	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	Mean of CpG
Week 10	2·75	2·50	3·25	3·75	1·75	1·75	3·00	2·68
Week 49/50								
AL	2·50	2·25	2·75	3·75	1·75	0·75	2·50	2·32
CER	4·25	1·00	3·50	4·25	1·25	1·00	1·25	2·36
IER-RF	2·25	1·75	2·25	3·25	1·25	1·25	1·50	1·93
IER-R	5·00	0·75	2·75	4·00	2·00	0·50	0·75	2·25
Week 81/82								
AL	5·25[a,b]	4·00	3·75	6·50	3·75	1·25	4·00	4·07
CER	1·50[a]	1·50	2·00	3·25	1·00	1·00	1·75	1·71
IER-RF	8·33[b]*	6·33*	5·00	6·67	3·67	1·67	4·00	5·10
IER-R	5·00[a,b]	3·50	3·75	7·00	2·50	2·50	3·50	3·96

AL, *ad libitum*; CER, chronic energy restriction; IER-RF, intermittent energy restriction-refed; IER-R, intermittent energy restriction-restricted.
[a,b] Values with unlike superscript letters were significantly different among energy-restricted groups at the same time point.
* Statistical significant changes in the IER-RF group by ageing.

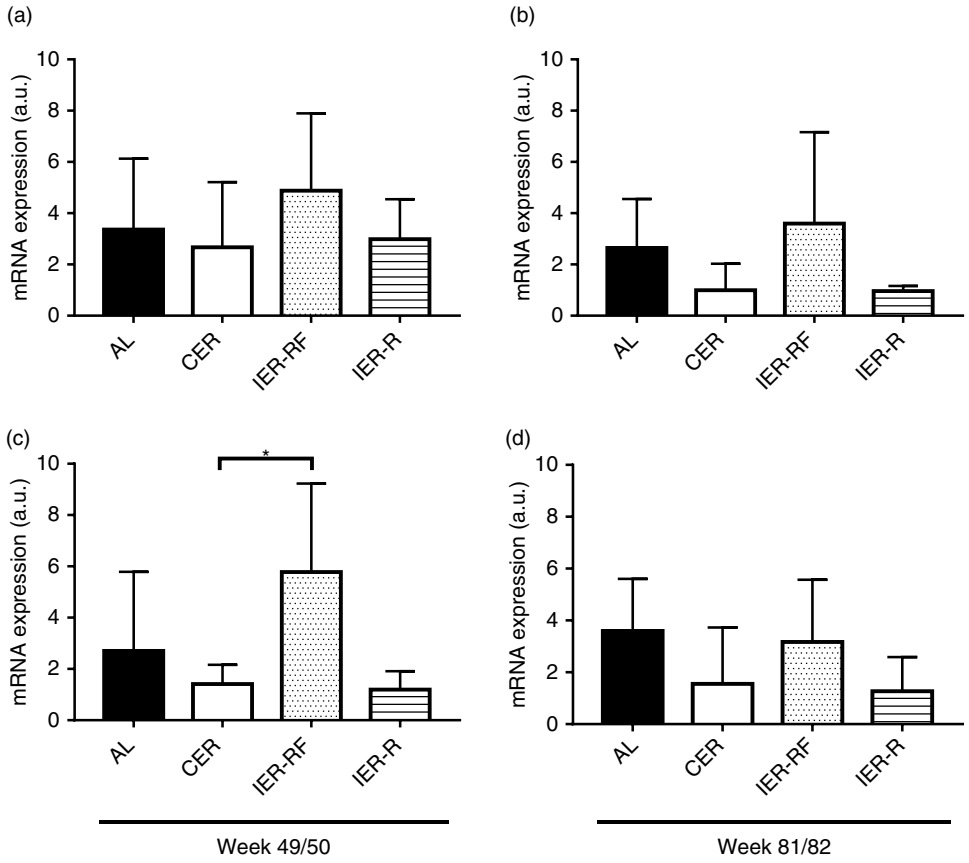

Fig. 4. Effects of different types of energy restriction on mRNA expression levels of the adiponectin receptor 1 (*AdipoR1*) gene at week 49/50 (a) and at week 81/82 (b) and of the leptin receptor overlapping transcript (*Leprot*) gene at week 49/50 (c) and at week 81/82 (d). a.u., Arbitrary units; AL, *ad libitum*; CER, chronic energy restriction; IER-RF, intermittent energy restriction-refed; IER-R, intermittent energy restriction-restricted. Values are means, with standard deviations represented by vertical bars. * $P < 0·05$.

two genes in relation to the effects of ER in the prevention of MT development. To the best of our knowledge, the present study is the first one to report the effects of two different types of long-term ER (CER *v.* IER) on the methylation levels of *AdipoR1* and *Leprot* genes using a mouse model.

Adiponectin was shown to have protective effects on MT development, and it is regulated by *AdipoR1*. Therefore, methylation levels of *AdipoR1* may play a role in MT development by regulating *AdipoR1* gene expression levels and serum adiponectin concentrations. In the present study, compared with AL and IER groups, higher methylation levels of *AdipoR1* in the CER group were accompanied by both lower BW and MT occurrence. In this context, previous studies showed that compared with the AL group, serum adiponectin levels were higher in

ER groups, which had lower BW and MT occurrence rates[3,38]. Although there are no similar previous studies to compare our present findings with, the effects of other interventions on the methylation levels of adiponectin and leptin signalling-related genes were previously reported[5,49,50]. For example, it was reported that 36 h of fasting increased methylation levels in the promoter region of the adiponectin gene extracted from subcutaneous adipose tissue of young men[51]. Likewise, a different study conducted by Jiao *et al.* reported a decrease in the methylation levels of the adiponectin gene in visceral fat pad tissue of mouse pups whose mothers were fed a high-fat diet during gestation and lactation in comparison with mouse pups from low-fat fed mothers[52]. However, increased methylation levels of both adiponectin and leptin receptor genes were reported when the pregnant mothers were fed with a high-fat diet containing 60 % energy from fat during the late gestation period in the mouse model, while methylation of the leptin gene was decreased in the visceral fat tissue of the offspring[49]. Another study demonstrated that the methylation levels of adiponectin itself were increased in adipocyte tissue of mice fed a high-fat diet for 20 weeks[50]. In addition, higher methylation levels of the adiponectin gene in DNA isolated from either blood or adipose tissue of humans with high BMI were reported[5]. Non-significant effects of dietary interventions on methylation levels of *AdipoR1* or adiponectin receptor 2 genes in liver tissues of rats were also demonstrated using methylation-specific PCR[53]. Individual analysis of methylation levels at CpG sites was also examined in the present study. CpG2 and CpG3 sites of CER mice had significantly higher methylation levels than did the AL group. This result suggests that methylation levels of CpG2 and CpG3 may play more critical roles in the effects of ER in metabolic or pathophysiological activities and further study should identify their specific roles.

In general, in the present study, methylation levels were found to be lower compared with previous studies. However, there are other studies, which also report similar methylation levels in a variety of genes including the leptin gene in mice[54–56]. In these studies, average methylation levels of promoter regions of genes of interest ranged between 1 and 3 %[55,57]. Hence, physiological relevance of the methylation levels of the *AdipoR1* gene was studied by measuring mRNA expression levels of the gene itself. The CER group which had the highest methylation level of the *AdipoR1* gene had the lowest mRNA expression level of the *AdipoR1* gene itself. Similarly, although the methylation levels of the *AdipoR1* gene were lower in the AL group compared with the CER groups at week 81/82, mRNA expression levels of the *AdipoR1* gene in the AL group were higher. These results show that in general, there is a negative correlation between the methylation and mRNA expression levels. Therefore, even though the methylation levels of *AdipoR1* gene might seem to be lower (about 5 %), its physiological function might be significant for *AdipoR1* gene at week 81/82.

Leptin is another adipokine that regulates energy balance and food intake[31]. The metabolic functions of leptin and its receptors (ObRs) are regulated by a small transcription factor called *Leprot*. Recent studies claimed that *Leprot* silencing led to an increase in leptin signalling. Therefore, regulators of the receptors have become potential targets for analysis in order to gain a clearer understanding of the leptin and ObR signalling pathway[42]. In this context, epigenetic modifications of ObR gene could be important to better identifying the roles of leptin signalling in the preventive effects of ER on MT development.

Methylation level of CER was approximately two-fold lower compared with other dietary groups, although this difference did not lead to significant results. Lower methylation levels of *Leprot* in the CER group are in accordance with previous findings that increased leptin concentration was associated with enhanced proliferation in *in vitro* studies with human BC cells and with increased MT/BC development *in vivo* studies[3,37,38]. Here, we suggest a mechanism where lower methylation of the *Leprot* gene increases *Leprot* gene activation, which inhibits leptin receptor activation. Therefore, MT growth rate might be slower in the CER group in comparison with the others due to lower activation of leptin signalling. However, mRNA expression levels of the *Leprot* gene were not correlated with methylation levels of the gene. This might be due to the influence of the other post-transcriptional and epigenetics factors on *Leprot* gene. These factors may confound the effects of methylation for *Leprot* gene in mammary fat pad tissue. Related studies have reported contradicting results in terms of the leptin and/or leptin receptor methylation levels under the influence of dietary factors. For example, although some studies reported higher methylation levels of leptin when mice were fed a HFD, others reported otherwise[56,58–60]. In similar human studies that support our findings, Houde *et al.* reported lower leptin methylation levels in blood and adipose tissue of subjects with high BMI[5]. In addition, lower methylation levels of the leptin gene promoter region in humans with high BMI were reported after 8 weeks of a low energy diet[61].

When CpG islands of the *Leprot* gene in the promoter region were analysed individually, methylation levels of CpG1 in the CER group were three- to five-fold lower compared with this measurement in the other dietary groups at week 81/82; however, there was no significant difference among the dietary groups for the other CpG sites. This finding indicates that the CpG1 site of the *Leprot* gene may play an important and direct role in the leptin signalling pathway and MT development.

Effects of ageing on the methylation levels of *AdipoR1* and *Leprot* genes were also studied. In all dietary groups with the exception of *Leprot* in the CER group, the methylation levels were decreased from week 10 to week 49/50 and then dramatically increased at week 81/82. These results point out that methylation levels of these two genes change with ageing and they can be modulated by ER, which is one of the most effective strategies for healthy ageing. Interestingly, methylation levels of the *Leprot* gene were reversed by CER, which also decreased the MT occurrence rate in the present study (unpublished results, article under review). Therefore, the methylation levels of these two genes may be considered as ageing biomarkers and could be used as targets for dealing with ageing. In this context, changes in serum leptin levels with ageing have been reported in previous studies. Since adiponectin and leptin are interrelated, the results of the present study support that adiponectin

signalling-related genes may also take part in the ageing process. However, there are no studies specifically reporting roles of methylation levels of these two genes in ageing, although most studies reported increased methylation levels of leptin and/or adiponectin signalling-related genes with ageing[36,50,58–60]. However, the present study has limitations. Epigenetic mechanisms are known to include not only DNA methylation but also histone modifications, chromatin remodelling, structural and functional variance of histones and transcription of noncoding RNA. Inconsistent correlation between methylation levels and mRNA level might be related to these factors and should be further studied in order to better understand the roles of these two genes. Also, serum adiponectin and leptin levels could not be measured in the present study due to the small amount of serum samples that were available.

Conclusion

The present study demonstrated that methylation levels of *AdipoR1* and *Leprot*, which play important roles in adiponectin and leptin signalling pathways, can be modified by ER. When two different types of ER protocols were compared, CER had a more significant effect on methylation levels of both genes. This may explain why the CER group had lower MT incidence compared with either the AL or IER groups. In the present study, with the exception of the *Leprot* methylation level in the CER group, the methylation levels of both genes showed a similar trend in all dietary groups with ageing. They decreased at week 49/50, followed by a drastic increase at week 81/82. Methylation levels of the *Leprot* gene in the CER group decreased with ageing, while they increased in the other groups. These results indicate that methylation levels of *AdipoR1* and *Leprot* may play important roles in the protective effect of ER in MT development. Also, our results indicate methylation status of these two genes may be crucial as part of the protective effect of ER in the ageing process. Better understanding of the specific mechanisms of the protective effects of ER against cancer is vital in order to develop more efficient drugs and therapies as well as preventive strategies.

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Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114519002757

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