EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON METHYLATION LEVELS OF *ADIPOR1* AND *LEPROT* GENES IN BREAST CANCER MOUSE MODEL

by

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ABSTRACT

EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON METHYLATION LEVELS OF *ADIPOR1* AND *LEPROT* GENES IN BREAST CANCER MOUSE MODEL

Preventive effects of calorie restriction (CR) have been shown in many pathophysiological conditions including cancer using mouse models. In addition, animal and human studies showed the protective roles of CR in mammary tumor (MT) development. Adiponectin and leptin, adipokines, synthesized mainly in adipocytes have been stated to play roles in fatty acid oxidation, glucose regulation, and cancer cell growth. Both adiponectin and leptin signaling are facilitated by their receptors. Furthermore, epigenetic mechanisms like DNA methylation has a pivotal role in MT development. This mechanism can be changed by both the quality and quantity of dietary factors. Although the roles of many factors in the preventive effects of CR on MT development have been suggested, the exact molecular mechanism(s) of how CR prevents or delays MT development is not clear. In order to study the roles of methylation levels of *AdipoR1* and *LEPROT* genes in the effects of different types of CR, MMTV-TGF-α mice were divided into three different groups *Ad-Libitum* (AL), Chronic Calorie Restriction (CCR, 15 per cent CR) and Intermittent Calorie Restriction (ICR) group which was AL fed for three weeks (ICR-RF) followed by one week of 60 per cent restriction (ICR-R). The study period was from 10 until 82 weeks of age. Mice were sacrificed and tissues were collected at 10 (baseline), 49, 50 and 81, 82 weeks of age. Thereafter, methylation levels of the *AdipoR1* and *LEPROT* genes in mammary fat pad tissue was analyzed by pyrosequencing method. The methylation levels of *AdipoR1* and *LEPROT* genes for week 49/50 were similar for all dietary groups. Methylation levels of *AdipoR1* in AL, CCR, ICR-RF and ICR-R groups were 3.67 per cent, 13.83 per cent, 6.08 per cent, and 5.83 per cent at week 81/82, respectively. Significant difference was obtained between CCR and AL groups ($p<0.05$). On the other hand, methylation levels of *LEPROT* gene in AL, CCR, ICR-RF and ICR-R groups were 4.07 per cent, 1.71 per cent, 5.10 per cent, and 3.96 per cent at week 81/82, respectively. Adiponectin and Leptin gene expression levels were consistent with the methylation results.

ÖZET

MEME KANSERİ FARE MODELİNDE FARKLI ŞEKİLDE UYGULANAN KALORİ KISITLAMASININ *ADIPOR1* VE *LEPROT* GENLERİNİN METİLASYON SEVİYELERİ ÜZERİNDEKİ ETKİSİ

Kalori kısıtlamasının (KK) önleyici etkisi, kanser dahil bir çok patofizyolojik durum için, fare modelleri kullanılarak gösterilmiştir. Buna ek olarak, meme tümörü (MT) gelişiminde kalori kısıtlamasının koruyucu etkisi hayvan ve insan çalışmalarında gösterilmiştir. Çoğunlukla adipositlerde sentezlenen adiponektin ve leptin adipokinlerinin, glikoz regülasyonu, yağ asidi oksidasyonu ve kanser hücresi büyümesinde rol aldığı gösterilmiştir. Adipokin sinyallerinin her ikisi de kendi reseptörleri tarafından sağlanmaktadır. Ayrıca, DNA metilasyonu gibi epigenetik mekanizmalar da MT gelişiminde önemli bir role sahiptir. Bu mekanizma, besinsel faktörlerin kalitesine ve miktarına bağlı olarak değişebilmektedir. KK'nın, MT gelişimi üzerindeki koruyucu etkisinde bir çok faktörün rolü olduğu bilinmesine rağmen, KK'nın MT'nü nasıl önlediğinin veya geciktirdiğinin moleküler mekanizması net olarak belirlenmiş değildir. Farklı KK türlerinin etkisi dahilinde *AdipoR1* ve *LEPROT* genlerinin metilasyonlarının rolünü belirlemek amacıyla, MMTV-TGF-α fareleri, *Ad-Libitum* (AL), Kronik Kalori Kısıtlaması (KKK, yüzde 15 CR) ve üç hafta boyunca AL beslenen (AKK-NB) ve sonrasında bir hafta yüzde 60 kısıtlanan (AKK-K) Aralıklı Kalori Kısıtlaması (AKK) grubu olmak üzere üç farklı gruba ayrılmıştır. Çalışma periyodu, farelerin 10. haftasından 82. haftasına kadar devam etmiştir. Fareler, 10. (referans değeri), 49, 50. ve 81, 82. haftalarda sakrifiye edilmiştir ve dokular toplanmıştır. Meme dokusunda *AdipoR1* ve *LEPROT* genlerinin metilasyon seviyeleri, pyrosekans yöntemi kullanılarak ölçülmüştür. 49/50. haftalarda *AdipoR1* ve *LEPROT* genlerinin metilasyon seviyeleri tüm gruplar için benzerdir. *AdipoR1* geninin 81/82. haftadaki metilasyon seviyeleri, AL, KKK, AKK-NB ve AKK-K için sırasıyla yüzde 3.67, yüzde 13.83, yüzde 6.08 ve yüzde 5.83'tür. KKK ve AL grupları arasında anlamlı bir farklılık vardır ($p<0.05$). Bununla beraber, AL, KKK, AKK-NB ve AKK-K gruplarının *LEPROT* geninin 81/82. haftasındaki metilasyon seviyeleri sırasıyla, yüzde 4.07, yüzde 1.71, yüzde 5.10 ve yüzde 3.96'dır. Adiponektin ve Leptin gen ekspresyon seviyeleri, metilasyon sonuçları ile uyum içerisindedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ADIPOQ	Adiponectin gene
AdipoR1	Adiponectin receptor-1
AdipoR2	Adiponectin receptor-2
AL	*Ad-Libitum*
BMI	Body mass index
BW	Body weight
CCR	Chronic calorie restriction
CpG	Cytosine-phosphate-Guanine
CR	Calorie restriction
CVD	Cardiovascular disease
DIO	Diet-induced obesity
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide
FC	Food consumption
HDAC	Histone deacetylase enzyme
HFD	High fat diet
ICR	Intermittent calorie restriction
ICR-R	ICR - restricted
ICR-RF	ICR - re-feeding
LEP	Leptin gene
LEPROT	Leptin receptor overlapping transcript
LFD	Low fat diet
MFP	Mammary fat pad
MT	Mammary tumor
ncRNA	Non-coding RNA
ObR	Leptin receptor
PCR	Polymerase chain reaction
PD	Parkinson’s disease
SAM	S-adenosyl-L-methionine

1. INTRODUCTION

1.1. BREAST CANCER

Breast cancer is a cancer that develops from breast tissue and one of the most widespread disease among women that causes death in today's world. In 2012 World Cancer Research Fund Internatiol (WCRF) report, 1.7 million breast cancer cases were diagnosed while 521,900 deaths were reported due to breast cancer invasion [1].

There are many risk factors which may lead to breast cancer development such as genetic mutations, family history, hormonal status, stress, radiation as well as lifestyle factors including obesity and consumption of tobacco and alcohol [2-5]. In this context, it was reported that the roles of genetic mutations in breast cancer development gain importance [6], nearly 40 per cent of human breast cancers have p53 mutation and about 10 per cent of the cases has PTEN gene mutation. For example, mutation in BRCA1 and BRCA2 genes are the most widespread inherited breast cancer genes with prevalence within the range of 2 – 3 per cent. DNA strand break repairment is facilitated by these genes. BRCA2 related tumours are more likely to be estrogen receptor (ER)-positive, and human epidermal growth factor receptor 2 (HER2)-negative as opposed to BRCA1 which are mainly triple negative (ER-, PR-, HER2-) [7, 8]. On the other hand, life style may significantly affect breast cancer development. For example, previous studies with middle-aged women conducted by Allen *et al*. have reported that each 10 g/day increment in alchol consuption, there is a 9 per cent increase in the risk of breast cancer [9]. Moreover, increase in cigarette smoking enhances breast cancer risk by 9-16 per cent [10]. It is also revealed that obesity increases the breast cancer risk in postmenopausal women by 30 to 50 per cent [11]. Furthermore, previous studies have reported being physically active decreases breast cancer risk by 25-30 per cent [12, 13]. In addition, previous study conducted by Toledo *et al*. have reported the consumption of olive oil decreased breast cancer risk by 62 per cent [14].

1.2. BREAST CANCER AND CALORIE RESTRICTION

The epidemic of obesity is one of the major health problems in modern world. In 2013, approximately 2.1 billion people were reported to be overweight or obese woldwide [15]. Obesity is a metabolic disorder occurred by excessive adiposity which is usually resulted from higher calorie intake than needed. In order to measure obesity, Body Mass Index (BMI) is used which is calculated by;

$$BMI = {weight\ (kg)}\Big/{height\ (m^2)} \tag{1.1}$$

According to BMI calculation, who has BMI result equal or gretar than 30 defines as an obese [15]. Less physical activity, and high food consumption are the major causes of obesity in today's lifestyle. It is also known that obesity is a major risk factor for numerous disorders and diseases such as coronary health disease, type 2 diabetes, and all kind of cancers including breast cancer [16, 17]. Indeed, obesity itself is considered to be a metabolic disorder. In this context, it was reported that obesity causes an increase in breast cancer development risk in postmenopausal women by 30 to 50 per cent [11]. Furthermore, animal studies have reported that calorie restriction (CR) which is described as decrease in calorie intake without malnutrition have protective effects on variety of diseases like cardiovascular diseases, diabetes and experimental cancers including breast cancer [18-20]. There are two common types of CR methods applied in studies; Chronic Calorie Restriction (CCR) and Intermittent Calorie Restriction (ICR). Both methods have been shown to prevent breast cancer development especially ICR. For example, in animal studies, the MT incidence of MMTV-TGF-α mice in AL group was 50–80 per cent , whereas the MT incidence of mice included in CCR group was 27–44 per cent , however, 3-15 per cent of MT incidence rate was observed in ICR mice restricted similarly but in an intermittent manner [19, 21-24].

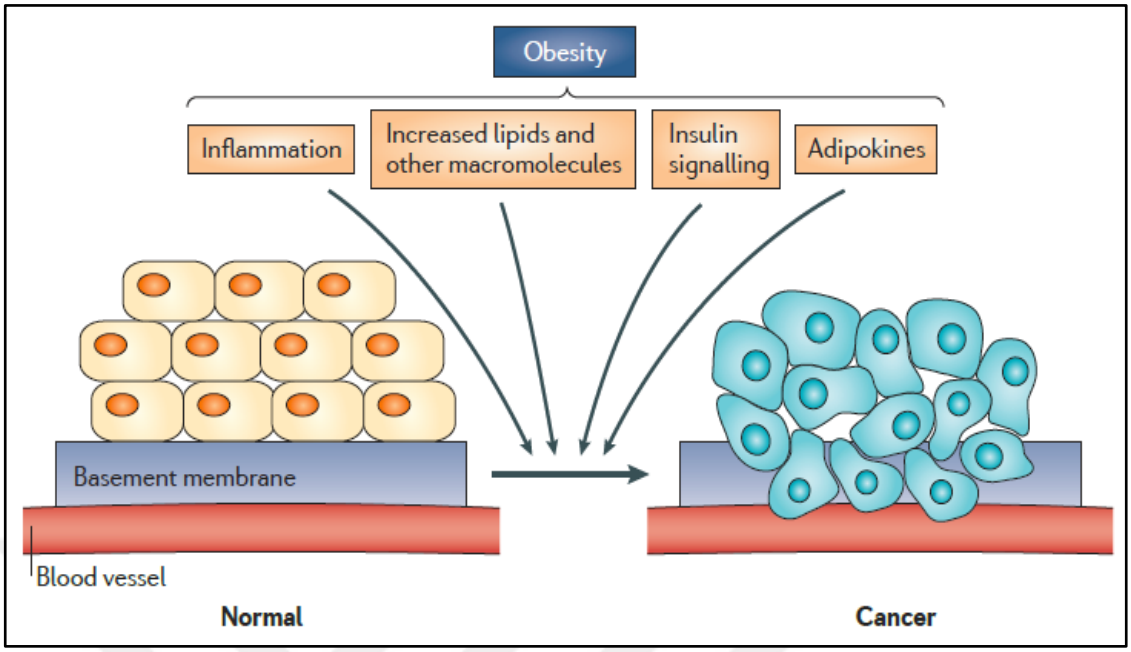

Figure 1.1. Effects of obesity on cancer development [16]

1.3. ROLES OF ADIPOKINES IN MAMMARY TUMOR DEVELOPMENT

1.3.1. Roles of Adiponectin Signaling Pathyway

Obesity is counted as a risk factor for some cancer types like breast and prostate and these obesity-related cancer risks are resulted from the different types of adipocytokines [25-28]. One of the significant adipokine in obesity-related cancers is adiponectin [29, 30]. Adiponectin, 30 kDa protein encoded by *ADIPOQ* gene is abundantly found in adipose tissue and it is composed of 244 aminoacids [26, 28, 31, 32]. Using cell culture and animal models, studies have revealed that adiponectin plays significant roles in growth and migration of cancer cell, insulin sensitivity, lipid metabolism and glucose regulation [29, 33-35]. Adiponectin has two receptors, Adiponectin Receptor-1 (AdipoR1) with 42 kDa and Adiponectin Receptor-2 (AdipoR2) with 43 kDa [25, 36, 37]. AdipoR1 is encoded by *AdipoR1* gene located on chromosome 1 at position 1q32.1 in human and chromosome 1 at position 1E4 in mouse, has higher affinity than AdipoR2, thus it has more definite role in breast cancer (Figure 1.2) [33, 38-40].

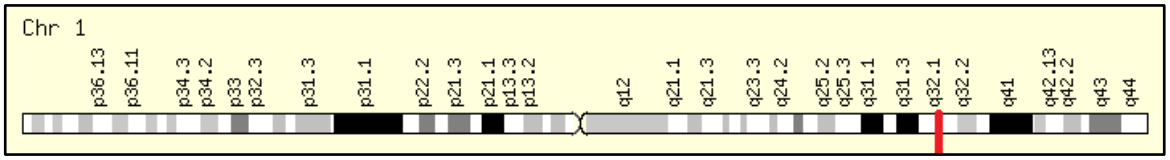

Figure 1.2. Genomic location of *AdipoR1* [41]

The concentrations of adiponectin is inversely correlated with BMI, thus low adiponectin levels in obese patients result in increased breast cancer risk [42-46]. The growth-inhibitory effect of adiponectin on breast cancer development has been reported in previous studies and this effect is mediated by AdipoR1 [36, 47-52]. For example, up to 40-66 per cent of growth inhibitory effect of adiponectin was reported in T47D, MCF- 7 and MDA-MB-231 breast cancer cell lines and inhibitory effect was through AdipoR1/R2 signaling pathways [47, 48, 53, 54]. In addition, roles of adiponectin in MT development was implied in both animal and human studies [19, 55]. In this context, mammary tumor mouse model study showed that lower serum adiponectin levels were observed in animal groups that had higher breast cancer incidence rate in comparison to the that had less MT incidence rate [19]. However, some studies reported serum adiponectin levels have not changed significantly between MT developed and MT free groups [56, 57]. In human, studies reported that women with high adiponectin levels had 65 per cent lower breast cancer risk compared to the control ones [2, 58].

1.3.2. Roles of Leptin Signaling Pathway

Leptin is another adipokine which is encoded by *LEPTIN (LEP)* gene and produced primarily in adipose tissue [59]. It acts as a neurohormone that regulate energy balance and food intake [60]. It is known that the physiological actions of leptin is controlled by Leptin Receptor (ObR) which is a single membrane-spanning receptor homologous to members of the class I cytokine receptor [60-62]. ObR is expressed in six isoforms and divided into three structural subgroups; long, short and secretory [62, 63]. Ob-Rb is a long isoform and Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf are short isoforms of ObR. ObR isoforms have the same sequnce of first intracellular 29 amino asids, except identical extracellular and transmembrane domains. The other isoform of ObR, Ob-Re, does not include intracellular and cytoplasmic domain [62, 64, 65].

Many studies reported promoting impacts of leptin on breast cancer development using cell culture and animal experiments [19, 61, 66, 67]. For example, incubation of T47D cells with leptin increased proliferation of cancer cells by 60-138 per cent [55, 60, 68]. In addition, mammary tumor mouse model study has shown that animal groups that had higher breast cancer incidence rate had higher serum leptin levels compared to the that had less MT incidence rate [19]. Besides, some studies reported no significant difference between MT developed and MT free groups for serum leptin levels [56]. In human studies, serum leptin levels of breast cancer patients are significantly higher than the healthy ones [69, 70]. Hence, examination of the roles of leptin and leptin receptor signaling pathway in great detail is required in order to better understand the nutritional effects on cancer development [60]. For this reason we investigated the roles of methylation of Leptin Receptor Overlapping Transcript (*LEPROT*) gene located on chromosome 1 at position 1p31.3 in human and chromosome 4 at position 4C6 in mouse, in this process, since regulation of leptin receptor activation by LEPROT has been shown previously (Figure 1.3) [60]. In this context, studies have reported negative regulation of leptin receptor cell surface expression level by LEPROT [71, 72]. Both *ObR* and *LEPROT* gene are transcribed under the control of the same promoter [73, 74]. Besides, some studies have revealed the effects of *LEPROT* gene 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 [72, 75]. As a matter of fact that previous studies claimed LEPROT is therapeutic target for the treatment of obesity [72, 76]. Regulators of the receptor may be more convenient target than the receptor, thus LEPROT might be potential target to obesity treatment [72].

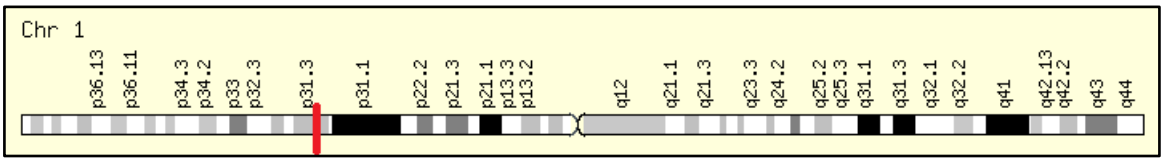

Figure 1.3. Genomic location of LEPROT [77]

1.4. EPIGENETICS

Epigenetics is a research area that becomes more significant last decades. In epigenetic mechanisms, gene expression is modified in a hereditary manner without changing DNA sequence, and these modifications affect how to read gene, and leads to gene on or off [78,

79]. The significant roles of epigenetic factors in the development of variety of diseases or metabolic syndromes such as neurodegenerative disease, cardiovascular disease (CVD) and cancer have been reported in recent studies [80-82]. Epigenetic modifications consist of DNA methylation, histone modification, and noncoding RNA associated gene regulation [82, 83].

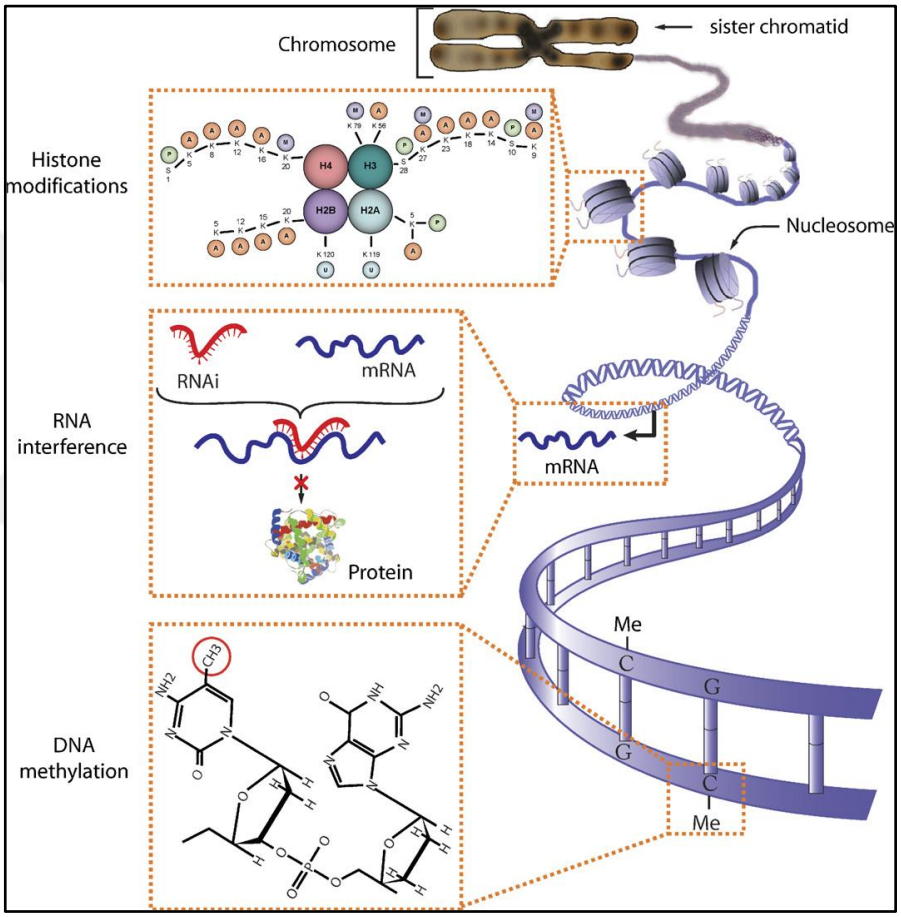

Figure 1.4. Three different epigenetic mechanisms [84]

1.4.1. DNA Methylation

DNA methylation has become the most investigated epigenetic mechanism due to its feasibility to measure and it exists in the X-chromosome stability, genomic imprinting, embryonic development, ageing and carcinogenesis [85-87]. It is occurred more than 98 per cent in Cytosine-phosphate-Guanine (CpG) sites of gene by covalenty transferring a methyl

group from methyl donor S-adenosyl-L-methionine (SAM) to the 5-carbon position of the cytosine via DNA methyltransferases (DNMTs) [85, 88, 89]. DNMTs are basicly divided into three; DNMT1, DNMT2 and DNMT3. More specifically, DNMT1 is defined as maintenance methytransferase because it prefers to methylate hemimethylated DNA, whereas DNMT2 is the smallest enzyme and its function in DNA methylation remains unclear. The third group of DNMTs is separated into three; DNMT3A, DNMT3B and DNMT3L. The methylation of unmethylated DNA is implemented by DNMT3A and DNMT3B and thus *de novo* methylation is performed by them. Conversely, DNMT3L is an inactive enzyme [85, 88-90].

Being mechanically understood and well conserved among species make DNA methylation more adventageous study in epigenetic modifications [88]. Gene transcription is affected by DNA methylation in the promoter region, therefore DNA methylation becomes one of the significant mechanisms of gene expression [91]. DNA methylation is divided into two; hypermethylation and hypomethylation. Hypermethylation means increase in global DNA methylation, whereas hypomethylation is defined as a decline in global DNA methylation. Roles of DNA methylation have been shown in diverse physiological and pathophysiological disorders such as cardiovascular diseases (CVD), aging, age-related neurological diseases like Parkinson's disease (PD), diabetes, and cancer including breast cancer [80, 81, 92-94]. In this context, some animal studies showed that change in DNA methylation can cause promotion of atherosclerosis [81, 95]. Furthemore, studies performed with PD patients revealed that risk factors of PD may be regulated by DNA metyhlation [80]. One of the previous study have reported the interaction between DNA methylation and type 2 diabetes, and revealed that increase in global DNA methylation levels are correlated with high insulin resistance [93]. Moreover, DNA methylation can cause to breast cancer development by means of hypermethylation of tumor supressor genes and/or hypomethylation of oncogenes [82, 96, 97]. Beside this, DNA methylation is known to be essential in breast cancer early detection; previous studies indicated the DNA methylation profiles might be a biomarker to make prediction and prevention of breast cancer easier [94, 98, 99].

1.4.2. Histone Modification

Histone modification is the second epigenetic mechanism. Gene expression can be regulated by modification of histones. H2A, H2B, H3 and H4 are core histones that form H2A and H2B dimers and H3-H4 tetramers [100]. The template of the genetic information for eukaryotics is chromatin which can be modified by histone proteins. Histone proteins are responsible to compact DNA and therefore regulate chromatin. Gene expression is regulated either positively or negatively through post-translational modifications of histone proteins [101]. Histone modifications include methylation, ubiquitylation, phosphorylation, acetylation and sumoylation of histones [100, 101]. Roles of histone modification in various physiological conditions including breast cancer have been shown in studies [102]. One of the previous study showed that in Huntington's disease, progressive neurodegeneration may be prevented by the inhibition of the histone deacetylase enzymes (HDACs) [102]. Moreover, some cancer studies claimed that HDAC inhibitors can be a potential treatment for breast cancer [103, 104].

1.4.3. Non-Coding RNA Associated Gene Regulation

Non-coding RNA (ncRNA) is known to be a functional RNA in which trascribed from DNA, however, can not be translated into protein. Even ncRNAs are not translated to protein, they are functional in the arrangement of gene expression. miRNA, siRNA, piRNA and lncRNA are included in epigenetic related ncRNAs [105, 106]. They can interact with methylation of DNA or histone modifications. ncRNAs have important roles in biological and pathological processes which include stem cell pluripotency, neurogenesis and oncogenesis [107-109]. Previous animal and human studies revelaed that miRNA alteration can be detected in mouse models of PD and PD patients' blood samples, therefore these changes in miRNA can be used as a biomarker [107, 108]. In addition to this, pivotal roles of ncRNAs in cancer-related pathways showed in previous studies and some studies claimed that for the detection of cancer, ncRNAs may be used as a biomarker [109, 110].

1.5. ROLES OF EPIGENETICS IN BREAST CANCER

Breast cancer is a multi factorial disease can be driven by both genetics and epigenetics factors. Epigenetic changes can be reasons of breast cancer, furthermore recent studies have claimed that epigenetic mechanisms might be used in prevention, prognosis and treatment of breast cancer [96, 111].

In the context of DNA methylation, more than 100 genes that are included in the regulation of cell-cycle, DNA repair as well as apoptosis are silenced by hypermethylation in mammary tumors or breast cancer cell line [112, 113]. Besides, previous studies have reported the roles of hypomethylation of oncogenes in breast cancer development [114, 115]. On the other hand, histone modification is the other epigenetic mechanisms in which associated with DNA methylation [116]. Post-translatinal modification of histones may play significant roles in breast cancer development. For example, previous study conducted by Yang *et al*. reported that in ER negative breast cancer patients, not only DNMTs but also HDACs are highly expressed in the promoter region of ER gene [117]. In addition, non-coding RNA associated gene regulation is another epigenetic modifications which may play significant roles in cellular regulation and apoptosis [96]. According to previous studies, abnormal expression of miRNAs are observed in breast cancer, for instance, the significant relation of higher expression of miR-21 with breast cancer was reported [118, 119].

Recent years, epigenetic modifications have been used as a biomarker for the detection of breast cancer. Previous studies have reported that an abnormal methylation of spesific genes can be detected in serum samples of breast cancer patients with sensitivity of 62-67 per cent and specificity of 75-87 per cent [120, 121]. Moreover, previous studies suggested the differentiation of breast cancer cells are inversely related with the number of methylated CpG islands [111, 122]. Therefore, classification of breast cancer may be associated with DNA methylation pattern [96].

Epigenetic modifications gain an importance for treatment of breast cancer. Previous researches have revealed that DNA methylation inhibitors (DNMTi) might be used to activate tumor suppressor genes silenced by DNA methylation [96, 123]. Major DNMT inhibitors are 5-azacytidine and 5-aza-deoxycytidine that incorporate into DNA and then inhibits DNMT activities [96]. However, since these DNMT inhibitors are highly unstable,

more stable derivative, zebularine, has been developed [124]. Recent study performed by Billam *et al*. has reported the efficacy of zebularine as a DNMTi by treating two human breast cancer cell lines, MDA-MB-231 and MCF-7, with zebularine. The effectiveness of zebularine as a DNMTi and demethlating agent has been revealed in human breast cancer cell lines, since the inhibition of cell growth is occured in a dose and time dependent manner [124]. On the other hand, HDAC inhibitors (HDACi) may be a potential treatment for breast cancer. One of the previous studies has reported that in more than 50 per cent of patients with metastatic breast cancer, partial or complete response is developed by HDAC inhibitors [103]. Moreover, combined therapy with DNMTi and HDACi has also been shown to induce synergistic (re-)activation of silenced tumor-suppressor genes in breast cancer [125, 126]. In addition, the regulation of miRNAs has been claimed to be an another therapeutic targets in breast cancer. Previous study conducted by Yan *et al*. has stated that tumor growth was inhibited by the knockdown of miR-21 in MCF-7 and MDA-MB-231 cells, therefore miR-21 inhibitors can be used as a possible treatment for breast cancer [119]. Another study conducted on miR-200a reported that cell proliferation is inhibited by miR-200a, thus it can also be a potential therapy in breast cancer [127].

2. MATERIALS AND PRIMERS

2.1. MATERIALS

- DNA Isolation Kit from Tissue, Macherey-Nagel, Munich, Germany
- Quick DNA Universal Kit, Zymo Research
- 5-mC DNA ELISA Kit, Zymo Research
- EpiTect Bisulfite Kit, QIAGEN GmBH Hilden, Germany
- PyroMark PCR Kit, QIAGEN GmBH Hilden, Germany
- PyroMark Denaturation Solution, QIAGEN GmBH Hilden, Germany
- PyroMark Wash Buffer, QIAGEN GmBH Hilden, Germany
- PyroMark Annealing Buffer, QIAGEN GmBH Hilden, Germany
- PyroMark Binding Buffer, QIAGEN GmBH Hilden, Germany
- PyroMark Q24 Plate, QIAGEN GmBH Hilden, Germany
- Streptavidin Sepharose, General HealthCare, London, UK
- PyroMark Gold Q24 Reagents, QIAGEN GmBH Hilden, Germany
- PyroMark Q24 Cartridge, QIAGEN GmBH Hilden, Germany
- Costar Plates, Sigma Aldrich, St. Louis, U.S.A.
- Thermowell Sealing Tape, Sigma Aldrich, St. Louis, U.S.A.
- DNA Ladder 100 bp, New England Biolabs
- Gel Loading Dye (6X), New England Biolabs

2.2. PRIMERS

- Adiponectin Receptor 1 (AdipoR1), QIAGEN GmBH Hilden, Germany

Table 2.1. *AdipoR1* Primer

Number of CpG sites	3
Sequence to Analyze	TCGGTCGGGAAGTTGATGGAGACTCGA
Sequence After Bisulfite Treatment	TYGGTYGGGAAGTTGATGGAGATTYGA
Nucleotide dispensation order	ATCGTCGAGATGAGTGAGATCG

- Leptin Receptor Overlapping Transcript 1 (LEPROT), QIAGEN GmBH Hilden, Germany

Table 2.2. *LEPROT* Primer

Number of CpG sites	7
Sequence to Analyze	CGTCAGGGGGCGGGCCGGGCTTTGGATTCGCGCGCCGGC
Sequence After Bisulfite Treatment	YGTTAGGGGGYGGGTYGGGTTTTGGATTYGYGYGTYGGT
Nucleotide dispensation order	GTCGCTCATGGTCGTCGTTGAGTCGTCGTCGTCG

2.3. INSTRUMENTS

- PyroMark Q24 MD, QIAGEN GmBH, Hilden, Germany
- Centrifuge, Spectrafuge 24D, Labnet International, Inc
- Spectrophotometer, NanoPhotometer, Implen, Munich, Germany
- Heater, Bioer
- Vortex, IKA
- Water Bath
- Gel Electrophoresis Equipments
- Micro Pipettes (1000, 200, 10 µl), Eppendorf

3. METHODS

3.1. ANIMAL AND STUDY DESIGN

In the current study, MMTV-TGF-α (C57/BL6) female mice were used. Main characteristics of these mice is the overexpression of human TGF-α which is a part of an epidermal growth factor receptor EGFR/ErbB cascade shown to be essential in the development of human breast cancer [128-130]. Therefore, these mice are prone to develop mammary tumor. Mice were attained from a colony in which maintained at Dr. Margot P. Cleary's lab at the Hormel Institute Medical Research Center, University of Minnesota and breeding colony was established at Yeditepe University Animal Facility.

At first, 10-week-old mice were randomly designated to three different dietary groups; *ad-libitum* (AL), chronic calorie restriction (CCR) and intermittent calorie restriction (ICR). The CCR group was applied to 15 per cent caloric restriction compared to AL groups from week 10 to week 82. The ICR group was subjected to 60 per cent caloric restriction compared to AL groups for one week, after restriction fed *ad-libitum* for following three weeks in a monthly cycle fashion, from week 10 until week 82 of mouse age. In ICR group, animals sacrificed at the end of one week of 60 per cent CR is referred as ICR-R (ICR-Restricted), while animals sacrificed at the end of three weeks of AL feeding period is referred as ICR-RF (ICR-Re-feeding). Diets (Altromin TPF1414) were purchased from Kobay AS (Ankara, Turkey).

Mice were sacrificed at designated weeks; week 10 as a baseline, week 49/50 and week 81/82. Mice were individually caged. Moreover, daily determination of food intakes and weekly determination of body weights were done. After overnight fasting, blood samples were collected and then mice were euthanized. Mammary fat pad (MFP) tissues were collected. After collection of tissues, a piece of MFP tissues were placed into a tube filled with formalin solution and pieces of tissues in formalin were delivered to the Pathology Department, Yeditepe University School of Medicine, for histopathological analyses. The residual tissues were stored -80°C until used. All animal studies were carried out after approval of and in compliance with the Committee of Animal Research Ethics of Yeditepe University.

3.2. MEASUREMENT OF DNA METHYLATION LEVELS OF *ADIPOR1* AND *LEPROT* GENES IN MFP TISSUES

3.2.1. DNA Isolation From MFP Tissues

- 25 mg of mouse mammary fat pad (MFP) tissues were cut into small pieces by lancet, and transferred into 1.5 ml microcentrifuge tube.
- Proteinase K solution was prepared.
- 180 µl of Buffer T1 and 25 µl of Proteinase K solution were added to tissue samples, and vortexed.
- Tissues with lysis solution were incubated at 56°C overnight, and during the inubation samples were shaked.
- After the incubation, samples were vortexed.
- 200 µl of Buffer B3 was added to each tube, and vortexed vigorously.
- Samples were incubated at 70°C for 10 minutes, and after the incubation vortexed briefly.
- In order to adjust DNA binding conditions, 210 µl of ethanol (96 per cent) was added, and samples were vortexed vigorously.
- NucleoSpin Tissue Column into a Collection Tube were prepared for each sample and then samples were placed into the column. Centrifugation of samples were performed at 11,000 x g for 1 minute. After centrifugation, flow-through was thrown away and tissue columns were put back into the collection tube.
- 500 µl of Buffer BW was added, and centrifugation of samples were performed at 11,000 x g for 1 minute. After centrifugation, flow-through were thrown away, then tissue column was put back into the collection tube.
- Buffer B5 was prepared. 600 µl of Buffer B5 was added to samples. Centrifugation of samples was performed for 1 minute at 11,000 x g. After centrifugation, flow-through was thrown away and tissue columns were put back into the collection tube.
- In order to eliminate remaining ethanol, column was centrifuged for 1 minute at 11,000 x g.
- NucleoSpin Tissue Columns were placed into a 1.5 ml microcentrifuge tubes. 75 µl of prewarmed (70°C) Buffer BE was added to each tube, samples were incubated at

room temperature for 1 minute. After incubation, centrifugation of samples was performed for 1 minute at 11,000 x g. First elution of DNA was obtained.

- Then, NucleoSpin Tissue Columns were put into a new 1.5 ml microcentrifuge tubes, in order to obtain second elution. After that 50 µl of prewarmed (70°C) Buffer BE was added to each tube, incubation of samples at room temperature was done for 1 minute, after incubation, centrifugation of samples was performed for 1 minute at 11,000 x g. Second elution was obtained.
- DNA concentration was measured using NanoPhotometer.
- Quality of DNA was checked by using gel electrophoresis.

3.2.2. Bisulfite Conversion

- Buffer BW was prepared by adding 30 µl of ethanol (96 per cent) and stored at room temperature.
- Buffer BD was prepared by adding 27 µl of ethanol (96 per cent) and buffer was stored at 4°C.
- 310 µl of RNase-free water was added to lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution, and solution was vortexed until the carrier RNA was dissolved. Dissolved Carrier RNA was stored at -20°C.
- Dissolved Carrier RNA was added to Buffer BL according to Table 3.1 in below;

Table 3.1. Volumes of carrier RNA and Buffer BL

Number of Samples	**1**	**4**	**8**	**16**	**24**	**48**
Volume of Buffer BL	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of Carrier RNA solution	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

- Bisulfite Mix was prepared by adding 800 µl of RNase-free water to each Bisulfite Mix aliquots, and vortexed until it was dissolved totally.
- Mixture of bisulfite reactions in PCR tubes were prepared according to Table 3.2.

Table 3.2. Components of bisulfite reaction

Component	**Volume per reaction (µl)**
DNA Solution (2 µg)	Variable* (maximum 20 µl)
RNase-free water	Variable*
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total volume	140

*The combined volume of DNA solution and RNase-free water must be total 20 µl.

- Bisulfite reactions were mixed.
- Thermal cycler program of bisulfite DNA conversion was indicated in Table 3.3.

Table 3.3. Bisulfite conversion thermal cycler conditions

Step	**Time**	**Temperature**
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite	20°C

- Following bisulfite conversion, tubes were centrifuged for a short time, then samples were placed into 1.5 ml microcentrifuge tubes.
- 560 µl of freshly prepared Buffer BL and carrier RNA mix was added to each tube and solutions were vortexed.
- Samples were placed into EpiTect spin columns and after that centrifugation of samples were performed at 13,000 rpm for 1 minute. After centrifugation, flow-

through was thrown away and then spin columns were put back into the collection tubes.

- 500 µl of Buffer BD was added to each sample, then incubation of samples were performed at room temperature for 15 minutes.
- After the incubation, spin columns were centrifuged at 13,000 rpm for 1 minute, flow-through was discarded, and spin columns were placed back into the collection tubes.
- 500 µl of Buffer BW was added to each spin column and centrifuged at 13,000 rpm for 1 minute, flow-through was discarded, spin columns were placed back into the collection tubes.
- Samples were again centrifuged at 13,000 rpm for 1 minute. After centrifugation, flow-through was discarded, then spin columns were put back into the collection tubes.
- Then, spin columns were placed into 2 ml collection tubes, and to remove any remaining liquids, centrifugation of spin columns were performed at 13,000 rpm for 1 minute.
- Spin columns were put into a new and clean 1.5 ml microcentrifuge tubes. after that incubation of samples were done at 56°C for 5 minutes to evaporate remaining liquid.
- Finally, spin columns were put into new 1.5 ml microcentrifuge tubes. 15 µl of Buffer EB was dispensed onto the center of each membrane. Purified DNA was obtained by centrifugation for 1 minute at 12,000 rpm.
- For the second elution, spin columns were placed into new 1.5 ml microcentrifuge tube, and 10 µl of Buffer EB was dispensed onto the center of each membrane. Centrifugation of samples were performed at 12,000 rpm for 1 minute, therefore second elution of purified DNA was obtained.

3.2.3. PyroMark Polymerase Chain Reaction

- Amplification of Bisulfite Converted DNA was performed by Polymerase Chain Reaction with biotinylated primer.
- Each required solutions were thawed and mixed according to Table 3.4.

Table 3.4. Reaction mix composition of PyroPCR

Component	**Volume per reaction**	**Final concentration**
PyroMark PCR Master Mix, 2x	12.5 µl	1X
CoralLoad Concentrate, 10x	2.5 µl	1X
Q-Solution, 5x (optional)	5 µl	1X
Primer	1 µl	0.2 µM/0.2 µM
RNase-free water	Variable	-
DNA	4 µl	
Total Volume (after adding template DNA)	**25 µl**	

- Reaction mix was prepared and gently pipetting. Mixture was dispended into PCR tubes.
- Bisulfite converted DNA was added to each PCR tube.
- Thermal cycler program was performed according to Table 3.5.

Table 3.5. Cycling protocol for PyroMark PCR master mix

			Additional comments
Initial PCR activation step	15 min	95°C	HotStartTaq DNA Polymerase is activated
3 step cycling: Denaturation	30 s	94°C	
Annealing	30 s	56°C	For bisulfite converted DNA
Extension	30 s	72°C	
Number of cycles	45		
Final extension	10 min	72°C	

- After PCR reaction, biotinylated PCR products were controlled by using agarose gel analysis.
- For pyrosequencing experiments, 10 µl of biotinylated PCR product was utilized.

3.2.4. Pyrosequencing by PyroMark Q24

3.2.4.1. Setting Up an Assay

- In order to create new assay for the PyroMark Q24 program, New CpG Assay was selected from the context menu, and file name was formed.
- Sequence to Analyze which is specific for gene of interest was typed.
- Dispensation Order was generated.
- Assays for both *AdipoR1* and *LEPROT* genes was created.

3.2.4.2. Setting Up a Run

- New run was selected from the context menu.
- Instrument method was selected.
- Plate was set according to analyze specific assays, and the sample ID was also created.

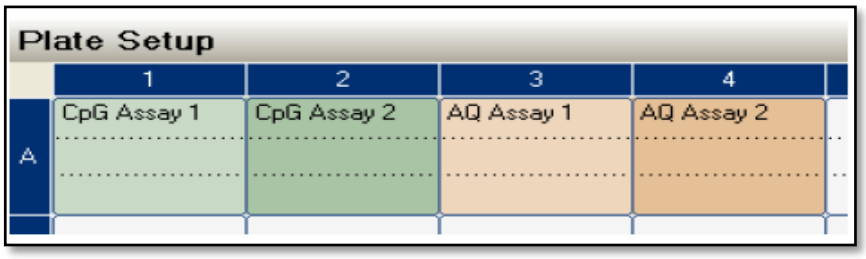

Figure 3.1. The plate setup of PyroMark Q24

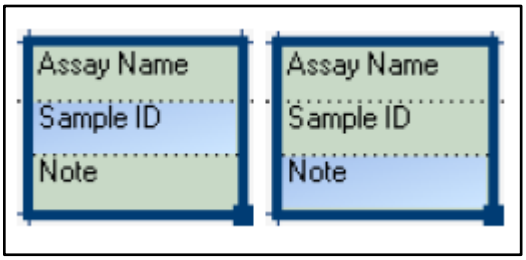

Figure 3.2. The well setup of PyroMark Q24

- By checking the Pre Run Information from the Tools menu, required volumes of enzyme mixture, substrate mixture and dNTPs was obtained which was used to prepare Cartridge.
- The run file was saved to the memory stick which was supplied by the system and used for the PyroMark q24 instrument.

3.2.4.3. Immobilization of the PCR Product to Beads

Biotinylated PCR products were immobilized on streptavidin-coated Sepharose beads.

- The bottle with streptavidin-coated Sepharose beads was shaked until homogenous solution was obtained.
- Streptavidin-coated Sepharose beads mixture was prepared with 2 µl of Streptavidin-coated Sepharose beads per sample, 40 µl of Binding Buffer per sample, and 28 µl of high purity water per sample, therefore the total volume was equal to 70 µl.
- Prepared Streptavidin-coated Sepharose beads mixture was dispensed to the PCR plate.
- 10 µl of well optimized, biotinylated PCR product was placed into wells and sealing of the PCR plate was done with strip caps.
- Plate was agitating at 14.000 rpm at room temperature for 10 minutes using a mixer.

3.2.4.4. Separation of the DNA Strands and Releasing of the Samples in PyroMark Q24 Plate

- 70 per cent ethanol was prepared by adding 35 ml 100 per cent ethanol and 15 ml high purity water.
- 5 ml Wash Buffer and 45 ml high purity water were used to prepare Wash Buffer.
- Five troughs were filled:
  i. Trough 1 was filled with 50 ml of 70 per cent ethanol
  ii. Trough 2 was filled with 40 ml of Denaturation Solution
  iii. Trough 3 was filled with 50 ml of Washing Buffer

iv. Trough 4 was filled with 50 ml of high purity water
v. Trough 5 was filled with 70 ml of high purity water

- Sequencing primer and Annealing Buffer mixture was prepared by adding 0,8 µl of Sequencing primer per sample, and 25,2 µl of Annealing Buffer per sample.
- 25 µl of sequencing primer mixture was dispensed onto the PCR plate.
- Vacuum switch was opened.
- Mixture contained sepharose beads was aspirated from all wells by tool and all beads were captured onto the filter probe tips.
- The tool was moved to Trough 1 and ethanol was flushed through the filter probes for 5 seconds.
- The tool was moved to Trough 2 and solution was flushed through the filter probes for 5 seconds.
- The tool was moved to Trough 3 and buffer was flushed through the filter probes for 10 seconds.
- The vacuum switch was turned off.
- Beads in the tool was released to the plate filled with sequencing primer and annealing buffer by shaking the tool.
- The tool was placed into the Through 4 which includes high purity water, and was agitated for 10 seconds.
- Then, vacuum switch was turned on and the tool was washed in the high purity water (Through 5)
- Vacuum pump was turner off.

3.2.4.5. Annealing of the Samples to a Sequencing Primer

- The plate including the samples was heated for 2 minutes with 80°C heated the holder.
- Then the plate with samples was cooled for 10 minutes.

3.2.4.6. Preparing PyroMark Q24 Gold Reagents

- Appropriate volumes of enzyme mixture, substrate mixture and the nucleotides was prepared and placed their specific region into the Cartridge.

3.2.4.7. Processing of the Run on PyroMark Q24 Instrument

- The filled cartridge was placed in the instrument with the label facing.
- The plate was placed in the heating block.
- The run was selected in the instrument's main menu and started.

3.2.4.8. Analysis of the Results

- The processed run file from the USB was moved to the computer which running the PyroMark Q24 Software.
- Then, anaysis for all wells was performed, pyrograms and methylation levels was recorded.

3.3. MEASUREMENT OF GLOBAL METHYLATION LEVELS

3.3.1. DNA Isolation From Liver Samples

At week 82 mice were euthanized, and liver tissues were collected to analyze global DNA methylation.

- 25 mg of liver samples were cut into small pieces by lancet, and put into 1.5 ml microcentrifuge tube.
- Proteinase K solution was prepared.
- 180 µl of Buffer T1 were added and then 25 µl of Proteinase K solution were added to tissue, and vortexed.
- Tissues with lysis solution were incubated at 56°C overnight, and during the inubation samples were shaked.

- After the incubation, samples were vortexed.
- 200 µl of Buffer B3 was added to each tube, and vortexed vigorously.
- Incubation of samples were performed at 70°C for 10 minutes. After the incubation samples vortexed briefly.
- To adjust DNA binding conditions, 210 µl of ethanol (96 per cent) was added, and vortexed vigorously.
- Samples were placed into the column and centrifugation of samples was performed at 11,000 x g for 1 minute. After centrifugation, flow-through was thrown away, then columns were put back into the collection tube.
- 500 µl of Buffer BW was added to samples. Then, centrifugation of samples were performed at 11,000 x g for 1 minute. After centrifugation, flow-through was thrown away, then columns were put back into the collection tube
- Buffer B5 was prepared and 600 µl of Buffer B5 was added to samples. After that, samples were centrifuged for 1 minute at 11,000 x g. After centrifugation, flow-through was thrown away and columns were put back into the collection tube.
- Samples in the column were centrifuged for 1 minute at 11,000 x g for removal of remaining ethanol.
- NucleoSpin Tissue Columns were put into a 1.5 ml microcentrifuge tubes. 75 µl of prewarmed (70°C) Buffer BE was added to each tube, incubation of samples were performed at room temperature for 1 minute, and then centrifugation of samples was performed at 11,000 x g for 1 minute. First elution of DNA was obtained.
- Then, NucleoSpin Tissue Columns were put into a new 1.5 ml microcentrifuge tubes, in order to obtain second elution. 50 µl of prewarmed (70°C) Buffer BE was added to each tube, incubation of samples were done at room temperature for 1 minute, and then samples were centrifuged for 1 minute at 11,000 x g. Second elution was done.

3.3.2. Global Methylation Measurement With 5-mC DNA ELISA Kit

DNA Coating:

- The required number of well strips were prepared.
- 100 ng of each DNA was placed into a PCR tube. After that volume was taken 100 µl with the addition of 5-mC Coating Buffer.

- Denaturation of DNA was performed at 98°C for 5 minutes in a thermal cycler. Then DNA was transferred instantaneously to ice and placed in ice approximately for 10 minutes.
- Total volume (100 µl) of denatured DNAs were added to plate. After that, foil was used to cover plate, then 2-hour incubation of plate was performed at 37 ºC.

Blocking:

- The buffer was discarded.
- Washing of each well was performed with 200 µl of 5-mC ELISA Buffer for 3 times. After each washing, buffer was discarded from the well.
- 200 µl of 5-mC ELISA Buffer was added to each well. After addition of buffer, foil was used to cover plate, then incubation of plate at 37 ºC for 30 minutes.

Antibody Addition:

- The buffer was discarded.
- An antibody mix including Anti-5-Methylcytosine and Secondary Antibody in 5-mC ELISA Buffer was prepared as stated in Table 3.6:

Table 3.6. Preparation of antibody mix

	Dilution	**Volume (µl)**	**Example (18 wells)**
5-mC ELISA Buffer	N/A	(# wells + 2) 100	2,000 µl
Anti-5-Methylcytosine	1:2,000	Buffer Vol. / 2,000	1 µl
Secondary Antibody	1:1,000	Buffer Vol. / 1,000	2 µl

- 100 µl of antibody mix was added to each well. Foil was used to cover plate, and incubation of plate was perofmed at 37ºC for 2 hour.

Color Development:

- At first, the antibody mixture was thrown away.
- Washing of wells were performed with 200 µl of 5-mC ELISA Buffer for 3 times.
- 100 µl of HRP Developer was added to each well, then color development was occurred in an hour at room temperature.

- By means of ELISA plate reader, the absorbance was measured at 405 nm.

3.4. STATISTICAL ANALYSIS

All statistical analyses of current study were performed by Prism Software Version 3.02. Results of experiments are shown as means ± SD. Chi-square test was used to analyze Mammary Tumor incidence data. In order to compare significance of differences among groups, first ANOVA test were done and then Post-Tukey's Test were performed. Because results of AL and CCR groups were not significantly different between two sequential time points at weeks 49 and 50; and also 81 and 82, data obtained from these two time points were pooled. On the contrary, these stated time points were displayed separately for ICR group of mice. When p value is equal or smaller than 0.05 ($p \leq 0.05$), it indicates statistical significance. "n" value refers to the number of individual mice in each group, and it is indicated in each figure legend.

4. RESULTS

4.1. BODY WEIGHT CHANGES

MMTV-TGF-α over expressed mice were enrolled in the calorie restriction study, then they were randomly assigned three different feeding groups; AL, CCR and ICR. Body weight (BW) of animals were measured weekly until the end of week 82. Average BW of AL, CCR and ICR groups were 27.68 (±2.9) g, 20.27 (±2.9) g, and 29.7 (±2.9) g at week 82, respectively. As it can be seen in the Figure 4.1, AL group had increasing BW, CCR group had almost stable BW change, whereas ICR group had variable body weight in compliance with restriction period (ICR-R week) or refeeding period (ICR-RF week). According to Figure 4.1, in ICR group, at week 13, 17, 21, 25, 29, 33, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, and 81 there was a sharp decrease in BW during restriction period and regain in BW starting with the refeeding period of three weeks. BW of CCR group was lower than others ($p < 0.0001$), while there was no significant difference between BW of AL and ICR groups. Mice food consumptions were measured daily. As expected CCR and ICR groups had lower food intake than AL group.

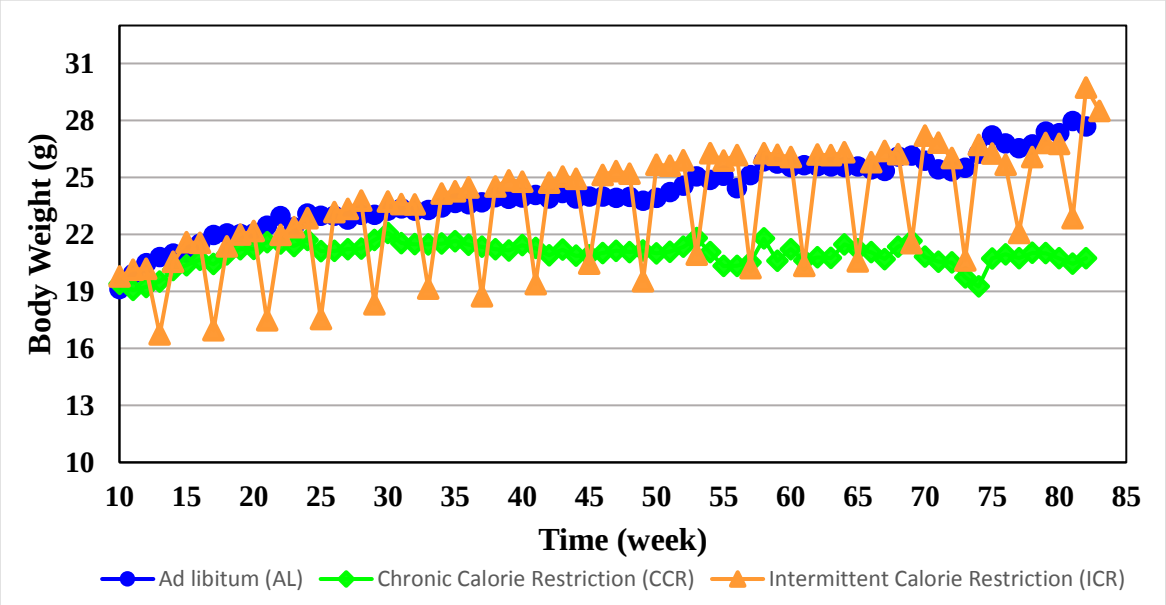

Figure 4.1. BW changing throughout the study

4.2. MAMMARY TUMOR DEVELOPMENT

In the present study MT incidence rate was calculated using only animals who lived after week 50 of their age. Mice sacrifized before week 50 were not included in MT incidence rate calculation because this model of animals do not usually developed MT before week 50 of their ages [19].

CR had significant effects on MT incidance rate ($p<0.05$). Although MT incidence rate was 39 per cent (13/33) in AL group this rate was 24 per cent (12/49) in ICR group. On the other hand, only 6 per cent (2/33) of mice in CCR group developed MT after week 50 of their age (Figure 4.2).

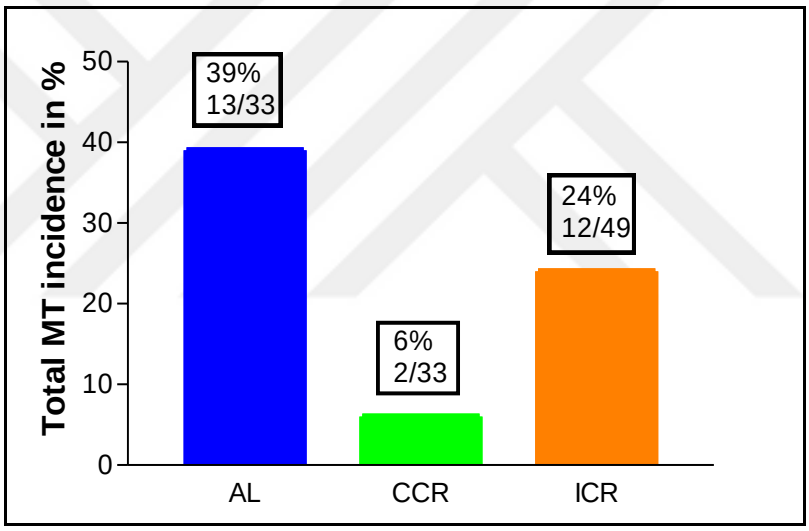

Figure 4.2. MT development in MMTV-TGF-α mice

4.3. METHYLATION LEVELS OF *ADIPOR1* AND *LEPROT* GENES IN MFP TISSUE

Before pyrosequencing analysis was performed, isolated DNA were run on the gel to confirm the quality of DNA isolated from MFP samples (Figure 4.3). Following DNA isolation, bisulfite treated DNA samples were amplified by PyroMark PCR kit and then results of the PCR was verified by agarose gel electrophoresis to see whether PyroMark PCR

step was succesfully worked. As been shown in Figure 4.4 for *AdipoR1* and Figure 4.5 for *LEPROT* genes.

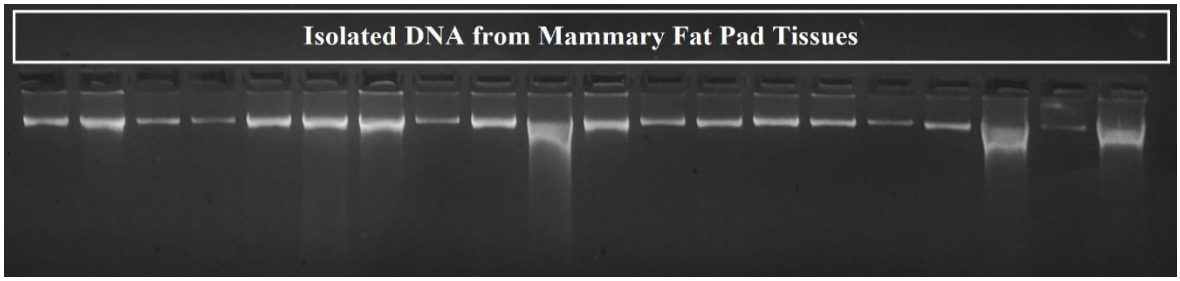

Figure 4.3. Representative gel imaging for isolated DNA samples from MFP tissues

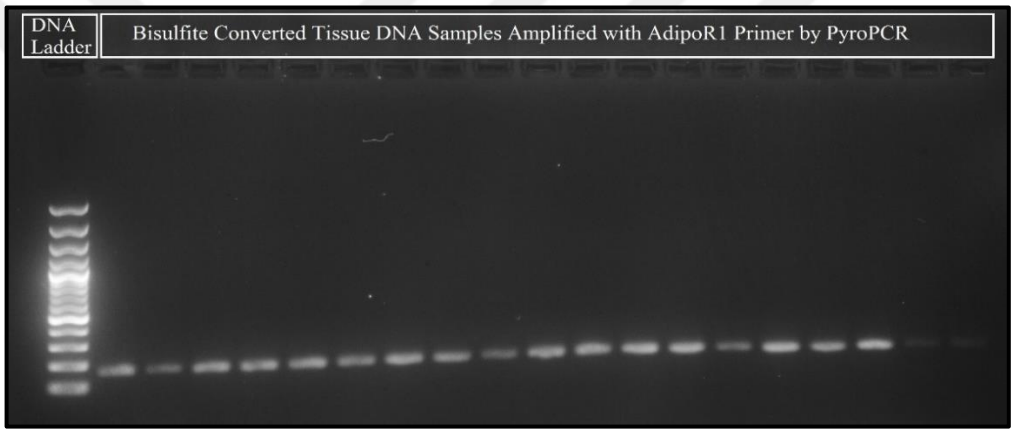

Figure 4.4. Representative gel imaging of PyroPCR results of bisulfite converted DNA for *AdipoR1* gene

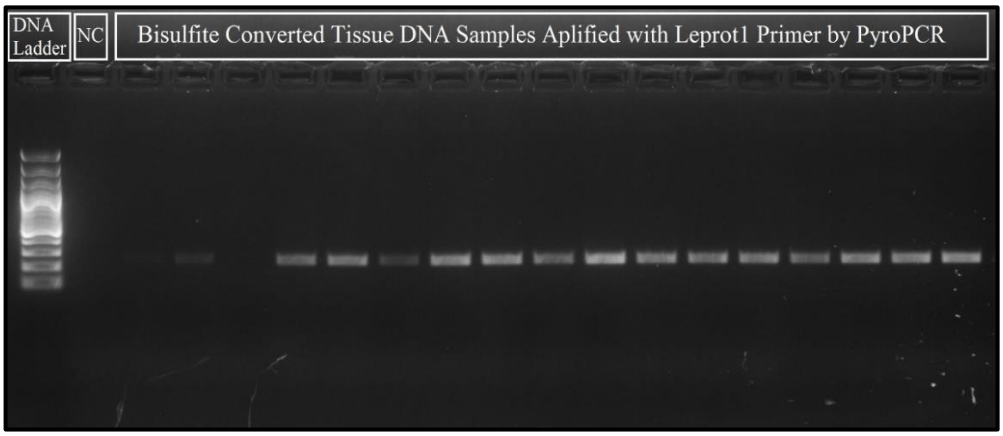

Figure 4.5. Representative gel imaging of PyroPCR results of bisulfite converted DNA for *LEPROT* gene

4.3.1. Changing in Methylation Levels of *AdipoR1* Gene in MFP Tissue with Two Different CR Types

The methylation levels of *AdipoR1* gene for week 49/50 were similar for all dietary groups. *AdipoR1* methylation levels of AL, CCR, ICR-RF and ICR-R at week 49/50 were 2.75 per cent, 2.33 per cent, 2.50 per cent, and 2.92 per cent, respectively (Figure 4.6). However, there was a significant difference between AL and CCR group for *AdipoR1* methylation level at week 81/82 ($p<0.05$, Figure 4.7). Methylation levels of *AdipoR1* of AL, CCR, ICR-RF and ICR-R were 3.67 per cent, 13.83 per cent, 6.08 per cent, and 5.83 per cent, respectively. Methylation levels of *AdipoR1* of CCR group was approximately four times higher than methylation levels of AL group. This difference was statistically significant ($p<0.05$). In addition, methylation levels of *AdipoR1* of CCR group was approximately two times higher than methylation levels of either ICR-RF or ICR-R at week 81/82 even though this difference was not statistically significant ($p>0.05$).

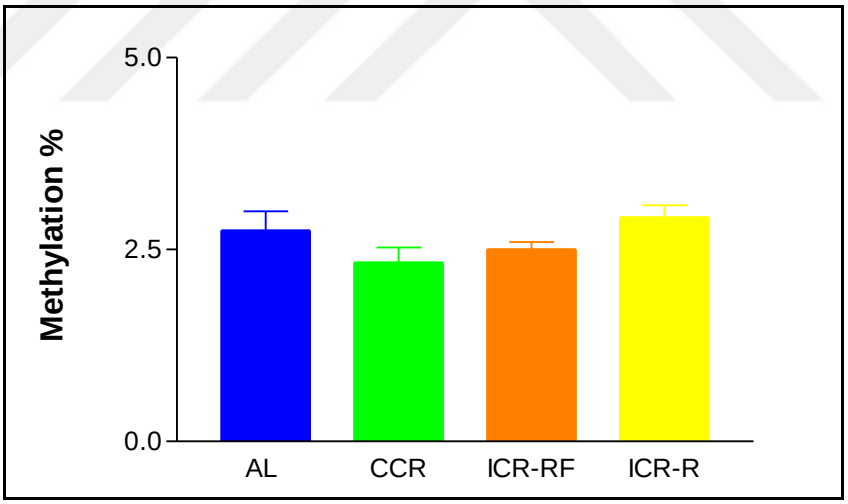

Figure 4.6. Changing in *AdipoR1* methylation levels in MFP samples of MMTV-TGF-α breast cancer mouse model with different CR types at week 49/50. "n" value represents samples taken from different animals (n= 3-4).

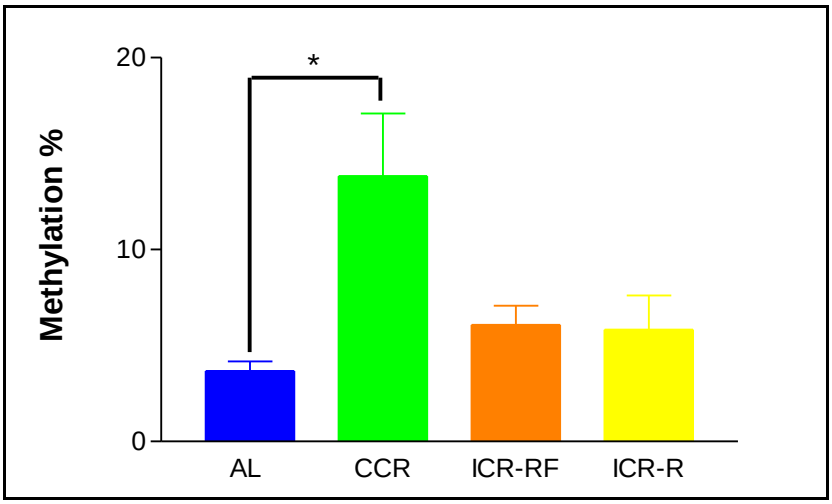

Figure 4.7. Changing in *AdipoR1* methylation levels in MFP samples of MMTV-TGF-α breast cancer mouse model with different CR types at week 81/82. "n" value represents samples taken from different animals (n= 3-4).

4.3.2. Changing in Methylation Levels of Individual CpG Islands of *AdipoR1* Gene in MFP Tissue with Two Different CR Types

In this study, methylation levels of individual CpG islands of *AdipoR1* gene was also measured. Promoter region of *AdipoR1* gene has three CpG islands. Methylation levels at CpG islands (CpG1, CpG2 and CpG3) were represented in pyrogram for each measurement. One of the examples of pyrogram can be seen in Figure 4.8. There was no statistical differences among the groups for the methylation levels of any of the CpG islands of *AdipoR1* gene at week 49/50 ($p>0.05$, Table 4.1). At week 81/82, methylation levels of CpG2 and CpG3 islands were statistically significant among the groups when it is analyzed by ANOVA ($p<0.05$, Table 4.1). In CCR group, the methylation levels of CpG2 and CpG3 islands were about three times higher than rest of the groups. Methylation levels of CpG1 island of *AdipoR1* gene in CCR group was five and two times higher than AL and ICR groups, respectively although, this difference was not statistically significant ($p>0.05$). There was no statistical differences among the groups for the methylation levels of any of the CpG islands of *AdipoR1* gene at week 49/50 ($p>0.05$)

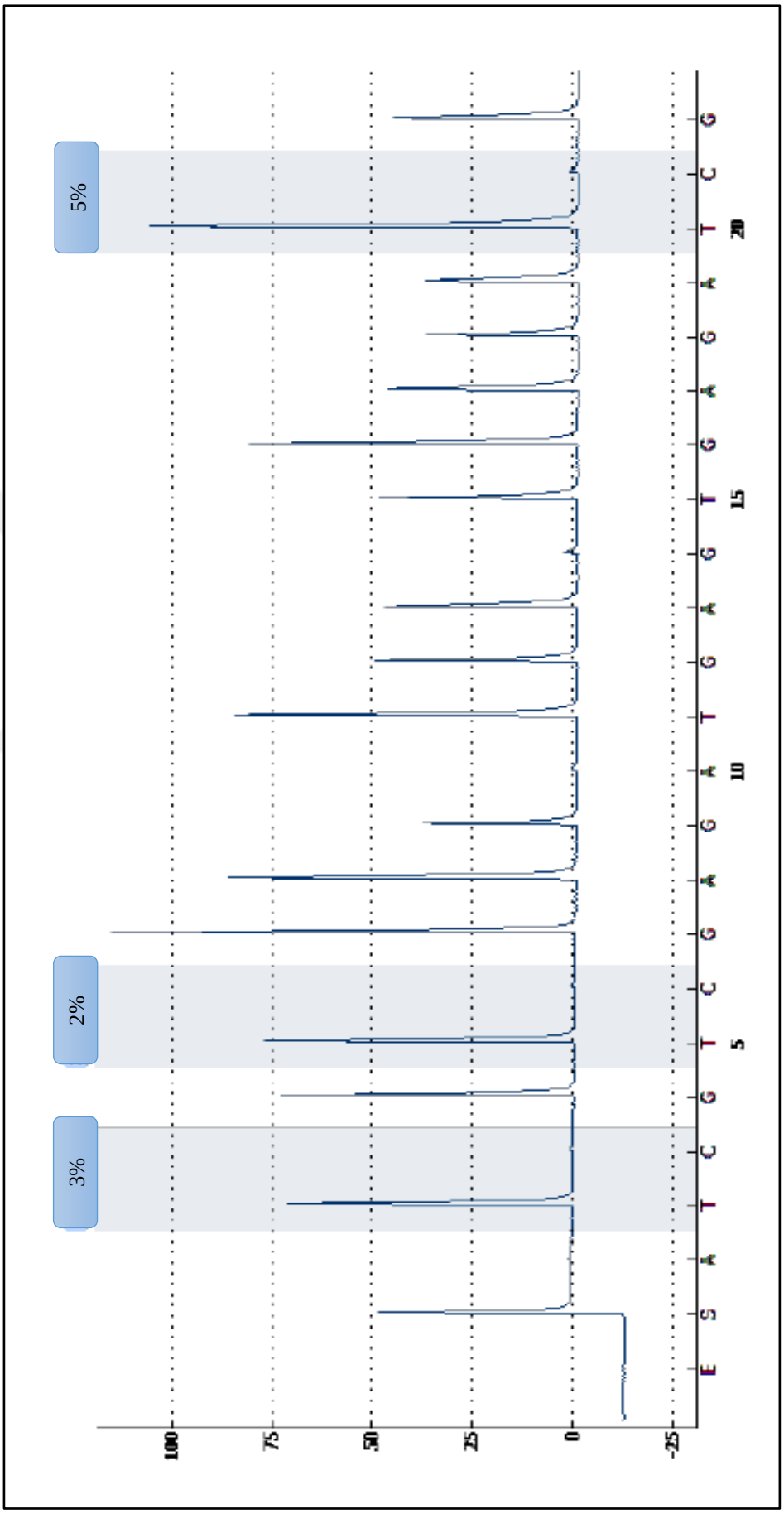

Figure 4.8. Representative pyrogram of *AdipoR1* gene

Table 4.1.Changing in methylation levels of CpG islands of *AdipoR1* gene in MFP tissues of MMV-TGF-α breast cancer mouse model with two different CR types

	AdipoR1	**CpG 1**	**CpG 2**	**CpG 3**	**Ave. of CpGs**
	week 10	5.50	4.25	5.00	**4.92**
week 49/50	***AL***	2.50	2.25	3.50	**2.75**
	CCR	2.00	2.33	2.67	**2.33**
	ICR-RF	1.75	2.25	3.50	**2.50**
	ICR-R	2.50	2.50	3.75	**2.92**
week 81/82	***AL***	3.33	3.00^{a}	4.67^{a}	**3.67**
	CCR	15.00	13.75^{b}	12.75^{b}	**13.83**
	ICR-RF	7.00	$5.25^{a,b}$	$6.00^{a,b}$	**6.08**
	ICR-R	7.00	$5.00^{a,b}$	$5.50^{a,b}$	**5.83**

*Significant difference was represented by values indicated with different letters in the same column. ‘‘n’’ value represents samples taken from different animals (n = 3-4).

4.3.3. Changing in Methylation Levels of *AdipoR1* Gene in MFP Tissue with Aging

In general, methylation levels of *AdipoR1* gene was decreased from week 10 to week 49/50, and then increased from week 49/50 to week 81/82. In AL group, methylation levels of *AdipoR1* was decreased by 44 per cent from week 10 to week 49/50, and then increased by 33 per cent from week 49/50 to week 81/82 (Figure 4.9). While methylation levels of *AdipoR1* was 2.75 per cent at the age of week 49/50, it was 3.67 per cent at 81/82 weeks of age in AL group. This change was not significant ($p>0.05$). In CCR group, conversely, changes in methylation levels of *AdipoR1* gene was statistically significant (Figure 4.10). Methylation levels was decreased by 52 per cent from week 10 to week 49/50, and increased by 493 per cent from week 49/50 to week 81/82. Furthermore, methylation levels was increased 181 per cent from week 10 to week 81/82 in CCR group ($p<0.05$, Figure 4.10). In CCR group, methylation levels of *AdipoR1* was 2.33 per cent at week 49/50, whereas it was 13.83 per cent at week 81/82. In ICR-RF group, *AdipoR1* methylation levels was decreased by 49 per cent from week 10 to week 49, and increased by 143 per cent from week 49 to week 81 (Figure 4.11). Increase in methylation levels from week 49 to 81 was statistically significant ($p<0.05$). While methylation levels of *AdipoR1* was 2.50 per cent at 49 weeks of age, it was 6.08 per cent at week 81 in ICR-RF group. In ICR-R group, methylation levels of *AdipoR1* was decreased by 40 per cent from week 10 to week 50, and increased by 99 per cent from week 50 to week 81/82 (Figure 4.12), although this increase was not statistically

significant (p>0.05). Methylation levels of *AdipoR1* in ICR-R group was 2.92 per cent and 5.83 per cent at the age of week 50 and week 82, respectively.

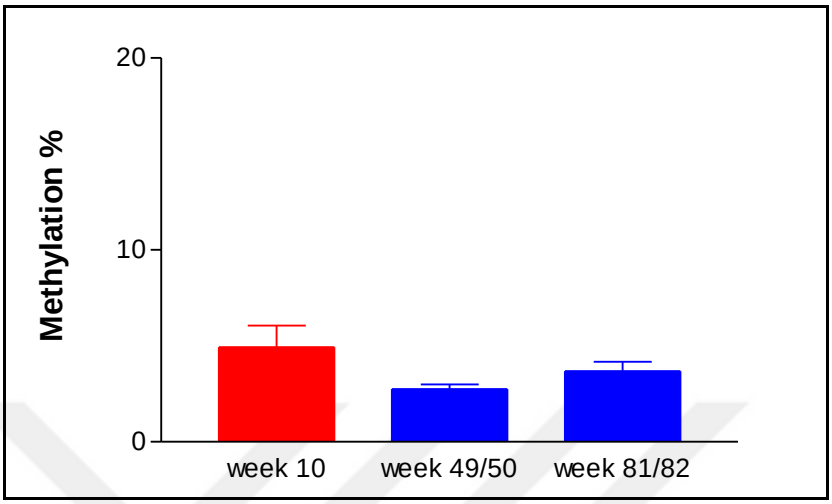

Figure 4.9. Changing in *AdipoR1* gene methylation levels in AL group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n= 3-4).

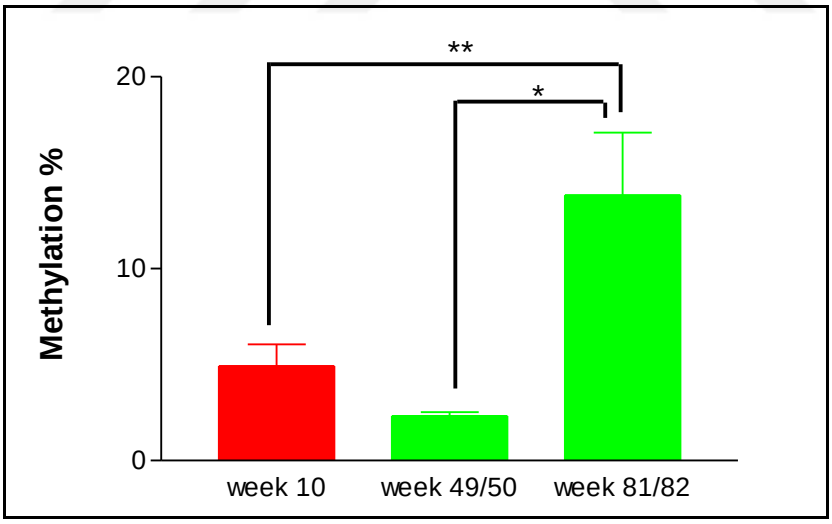

Figure 4.10. Changing in *AdipoR1* gene methylation levels in CCR group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

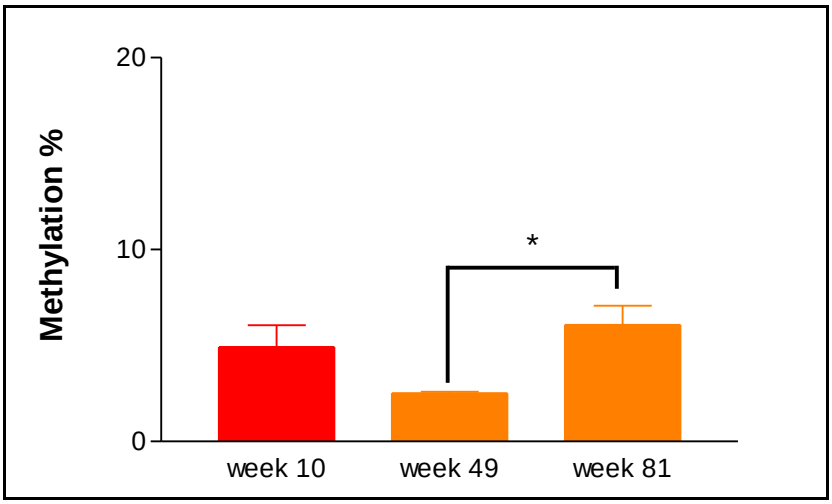

Figure 4.11. Changing in *AdipoR1* gene methylation levels in ICR-RF group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

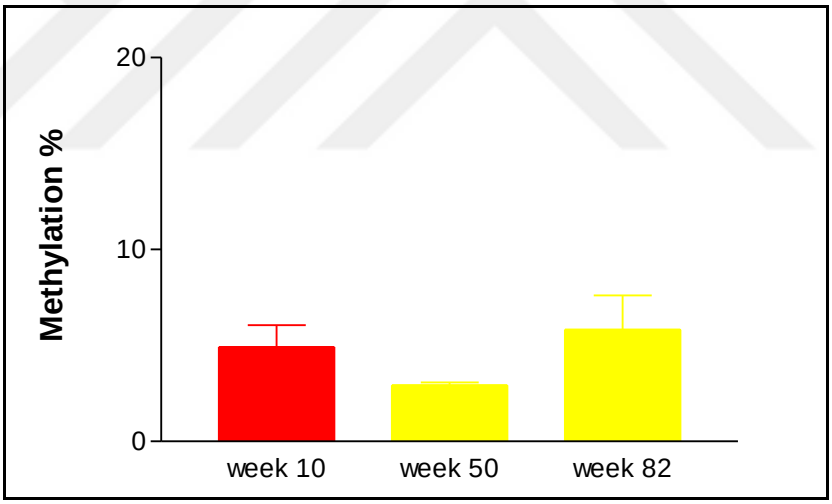

Figure 4.12. Changing in *AdipoR1* gene methylation levels in ICR-R group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

4.3.4. Changing in Methylation Levels of Individual CpG Islands of *AdipoR1* Gene in MFP Tissue with Aging

In addition, the effects of aging on methylation levels of specific CpG islands of *AdipoR1* gene in its promoter region was analyzed. There was an significant change in methylation levels of all three CpG islands (CpG1, CpG2, CpG3) in CCR group by aging. This change was statistically siginificant ($p<0.05$, Table 4.2). Moreover, in ICR-RF group, only the methylation level of CpG1 island was significantly increased by aging ($p<0.05$, Table 4.2). There was no change in CpG2 and CpG3.

Table 4.2. Changing in methylation levels of CpG islands of *AdipoR1* gene in MFP tissues of MMV-TGF-α breast cancer mouse model with aging

AdipoR1	**CpG 1**	**CpG 2**	**CpG 3**	**Ave. of CpGs**
week 10	5.50^{a}	4.25^{a}	5.00^{a}	4.92
CCR week 49/50	2.00^{a}	2.33^{a}	2.67^{a}	2.33
CCR week 81/82	15.00^{b}	13.75^{b}	12.75^{b}	13.83
week 10	5.50^{c}	4.25	5.00	4.92
ICR-RF week 49	1.75^{c}	2.25	3.50	2.50
ICR-RF week 81	7.00^{d}	5.25	6.00	6.08

*Only significantly changed groups were shown in this table. Significant difference was represented by values indicated with different letters in the same column. "n" value represents samples taken from different animals (n = 3-4).

4.3.5. Changing in Methylation Levels of *LEPROT* Gene in MFP Tissue with Two Different CR Types

The methylation levels of *LEPROT* gene for week 49/50 were similar in all groups (Figure 4.13). *LEPROT* methylation levels of AL, CCR, ICR-RF and ICR-R at week 49/50 were 2.32 per cent , 2.36 per cent , 1.93 per cent , and 2.25 per cent , respectively. Besides, methylation levels of CCR group of mice was lower than others at the age of week 81/82 (Figure 4.14). Methylation levels of *LEPROT* in CCR group was at least two fold lower than the other groups at week 81/82. Methylation levels of *LEPROT* gene were 4.07 per cent , 1.71 per cent , 5.10 per cent , and 3.96 per cent for AL, CCR, ICR-RF and ICR-R groups, respectively. But, this difference was not statistically significant ($p>0.05$, Figure 4.14).

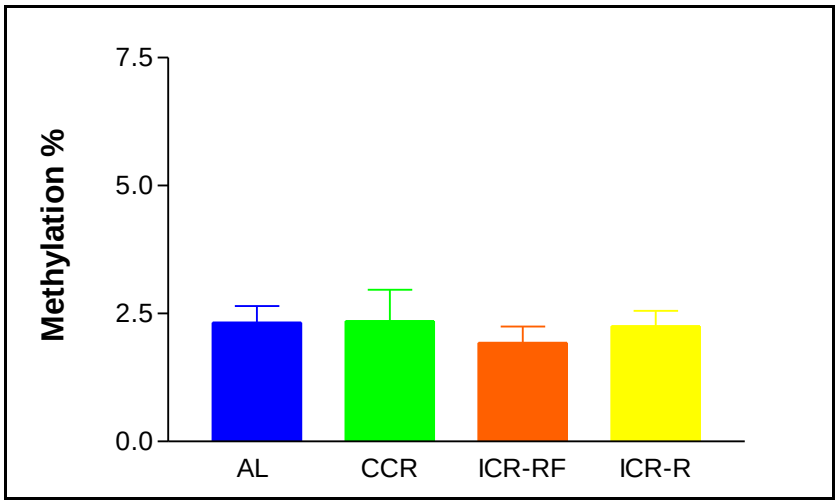

Figure 4.13. Changing in *LEPROT* methylation levels in MFP samples of MMTV-TGF-α breast cancer mouse model with different CR types at week 49/50 "n" value represents samples taken from different animals (n= 3-4).

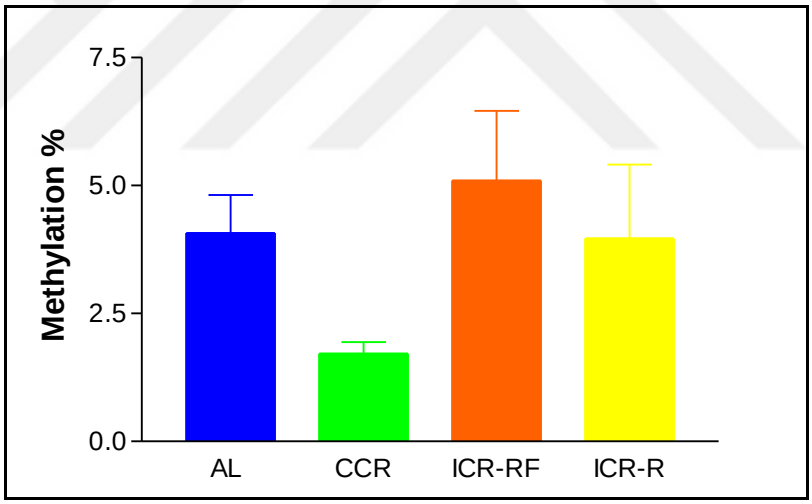

Figure 4.14. Changing in *LEPROT* methylation levels in MFP samples of MMTV-TGF-α breast cancer mouse model with different CR types at week 81/82 "n" value represents samples taken from different animals (n= 3-4).

4.3.6. Changing in Methylation Levels of Individual CpG Islands of *LEPROT* Gene in MFP Tissue with Two Different CR Types

Promoter region of *LEPROT* gene includes seven CpG sites. Methylation levels at CpG islands were represented in pyrogram for each measurement. One of the examples of

pyrogram can be seen in Figure 4.15. Among the seven CpG islands only the methylation levels of CpG1 island was significantly different among the dietary groups at week 81/82 ($p<0.05$, Table 4.3). The methylation levels of CpG1 island in CCR group was at least three to five folds lower compared to the rest of the groups (AL, ICR-RF and ICR-R) (Table 4.3).

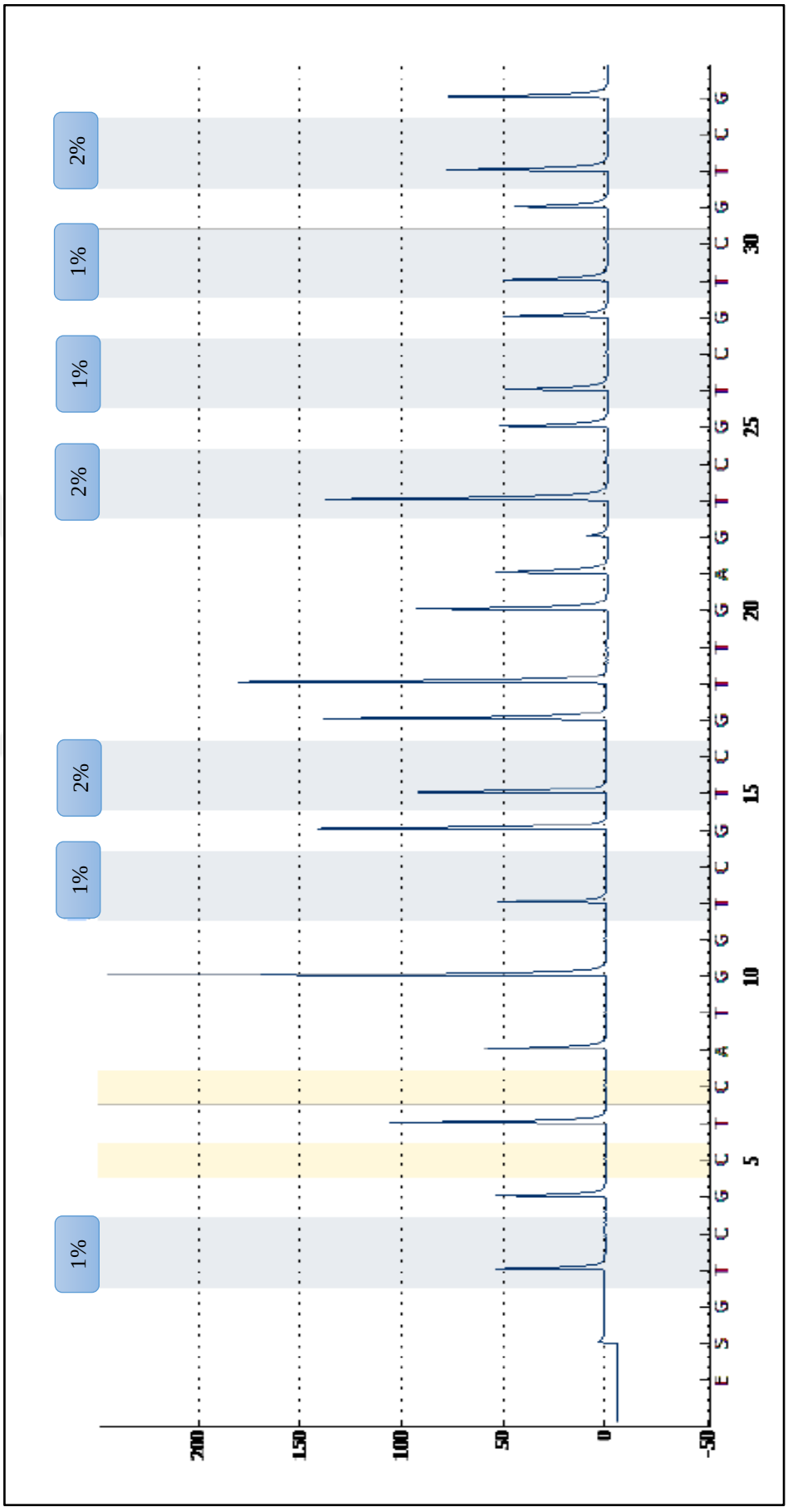

Figure 4.15. Represen tative pyrogram of LEPROT gene

Table 4.3. Changing in methylation levels of CpG islands of *LEPROT* gene in MFP tissues of MMV-TGF-α breast cancer mouse model with different CR types

	LEPROT	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**CpG7**	**Ave. of CpGs**
	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**
	CCR	4.25	1.00	3.50	4.25	1.25	1.00	1.25	**2.36**
	ICR-RF	2.25	1.75	2.25	3.25	1.25	1.25	1.50	**1.93**
	ICR-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**
	CCR	1.50[a]	1.50	2.00	3.25	1.00	1.00	1.75	**1.71**
	ICR-RF	8.33[b]	6.33	5.00	6.67	3.67	1.67	4.00	**5.10**
	ICR-R	5.00[a,b]	3.50	3.75	7.00	2.50	2.50	3.50	**3.96**

* Significant difference was represented by values indicated with different letters in the same column. "n" value represents samples taken from different animals (n = 3-4).

4.3.7. Changing in Methylation Levels of *LEPROT* Gene in MFP Tissue with Aging

The methylation levels of *LEPROT* gene was decreased from week 10 to week 49/50, and increased from week 49/50 to week 81/82 in AL, ICR-RF and ICR-R groups, but, the methylation level was gradually decreased in CCR group from week 10 to week 81/82. In AL group, methylation levels of *LEPROT* was decreased by 13 per cent from week 10 to week 49/50, and increased by 75 per cent from week 49/50 to week 81/82 (Figure 4.16). While methylation levels of *LEPROT* was 2.32 per cent at the age of week 49/50, it was 4.07 per cent at 81/82 weeks of age in AL group. In CCR group, methylation level was decreased by 12 per cent from week 10 to week 49/50, and decreased by 27 per cent from week 49/50 to week 81/82. Furthermore, methylation levels of *LEPROT* decreased by 36 per cent from week 10 to week 81/82 in CCR group (Figure 4.17). In CCR group, methylation levels of *LEPROT* was 2.36 per cent at week 49/50, whereas it was 1.71 per cent at week 81/82. In ICR-RF group, methylation levels of *LEPROT* gene was decreased by 28 per cent from week 10 to week 49, and increased by 164 per cent from week 49 to week 81 (Figure 4.18). While methylation levels of *LEPROT* was 1.93 per cent at 49 weeks of age, it was 5.10 per cent at the age of week 81 in ICR-RF group. In ICR-R group, the methylation levels of *LEPROT* gene was decreased by 16 per cent from week 10 to week 50,and increased by 76 per cent from week 50 to week 81/82 (Figure 4.19). Methylation levels of *LEPROT* in ICR-R group was 2.25 per cent and 3.96 per cent at the age of week 50 and week 82, respectively.

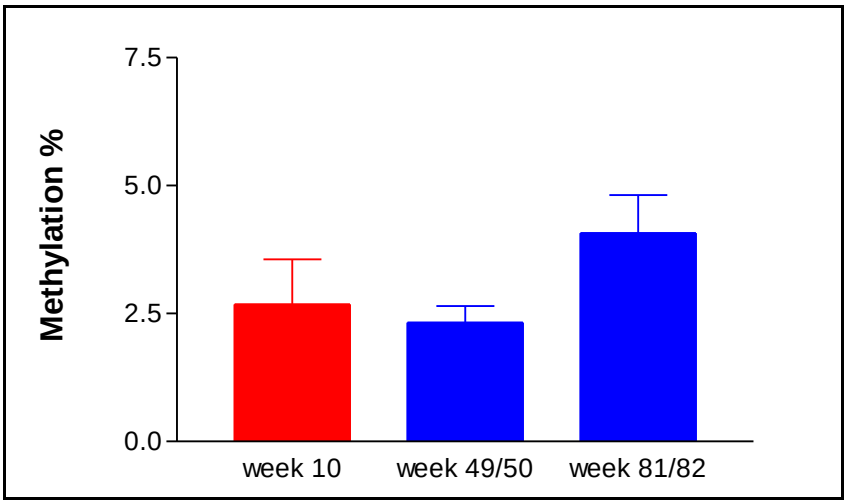

Figure 4.15. Changing in *LEPROT* gene methylation levels in AL group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n= 3-4).

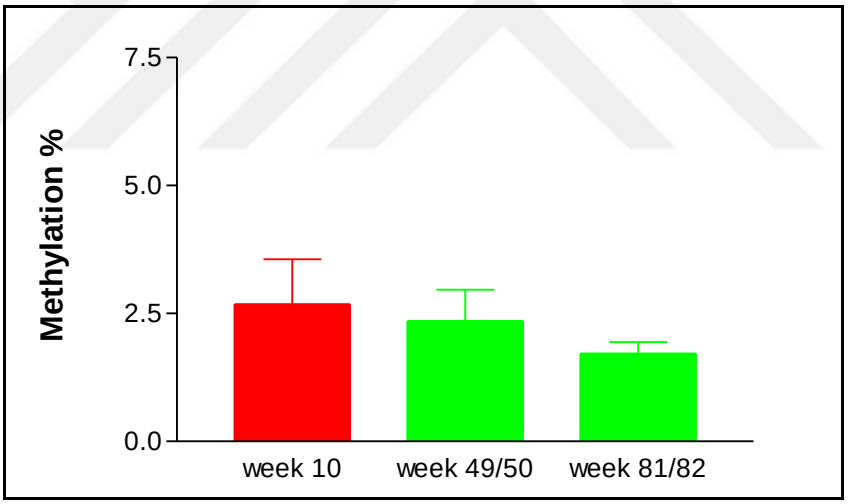

Figure 4.16. Changing in *LEPROT* gene methylation levels in CCR group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

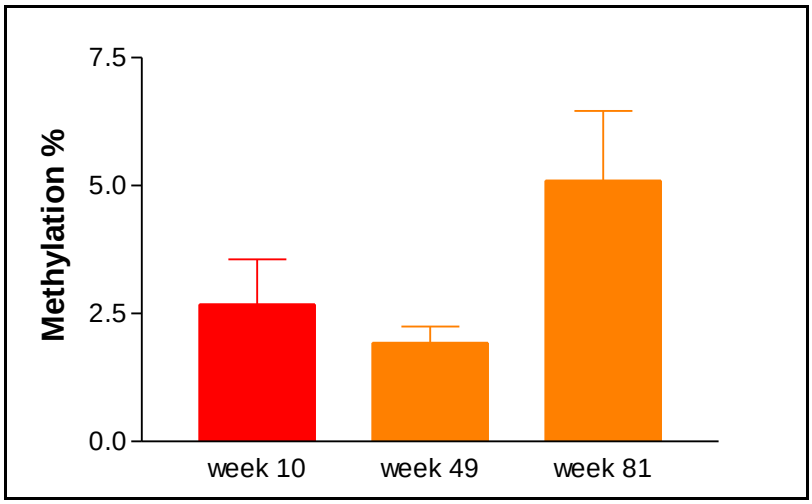

Figure 4.17. Changing in *LEPROT* gene methylation levels in ICR-RF group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

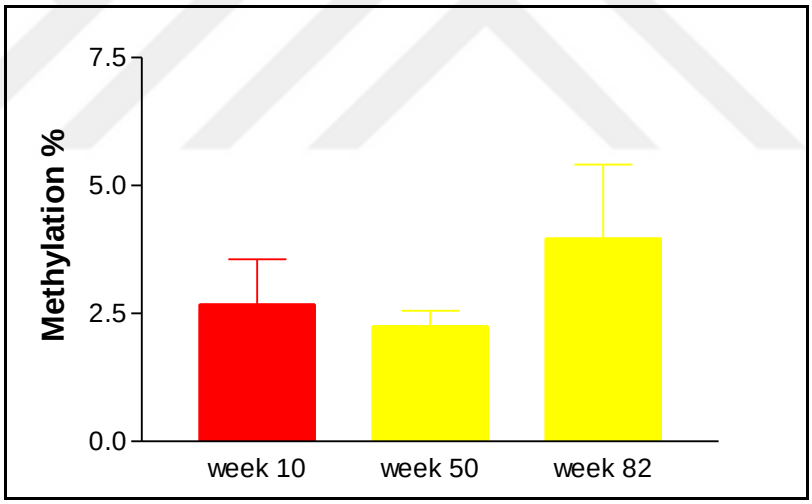

Figure 4.18. Changing in *LEPROT* gene methylation levels in ICR-R group MFP samples of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 3-4).

4.3.8. Changing in Methylation Levels of Individual CpG Islands of *LEPROT* Gene in MFP Tissue with Aging

The effects of aging on methylation levels of specific CpG islands of *LEPROT* gene in its promoter region was analyzed. The methylation levels of CpG1 and CpG2 islands were significantly increased by aging in ICR-RF group ($p<0.05$, Table 4.4). Two fold increase in methylation levels of CpG1 islands in AL group was observed, although this increase was not significant. On the other hand, there was a four fold decrease in the methylation levels of CpG1 island in CCR group from week 49/50 to 81/82. There was about two folds increase in the methylation levels of CpG4 islands in all groups except CCR group.

Table 4.4. Changing in methylation levels of CpG islands of *LEPROT* gene in MFP tissues of MMV-TGF-α breast cancer mouse model with aging

LEPROT	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**CpG7**	**Ave. of CpGs**
week 10	2.75[a]	2.50[a]	3.25	3.75	1.75	1.75	3.00	**2.68**
ICR-RF week 49	2.25[a]	1.75[a]	2.25	3.25	1.25	1.25	1.50	**1.93**
ICR-RF week 81	8.33[b]	6.33[b]	5.00	6.67	3.67	1.67	4.00	**5.10**

*Only significantly changed groups were shown in this table. Significant difference was represented by values indicated with different letters in the same column. "n" value represents samples taken from different animals (n = 3-4).

4.4. GLOBAL METHYLATION LEVELS

In mouse liver samples, global methylation analysis was performed using 5-mC Global Methylation ELISA Kit. Standard curve was created in order to calculate methylation levels in percent (Figure 4.20). Global methylation level of each dietary group was around 2 per cent at week 81/82 (Figure 4.21). Global methylation levels was not statistically different amongst the groups ($p > 0.05$).

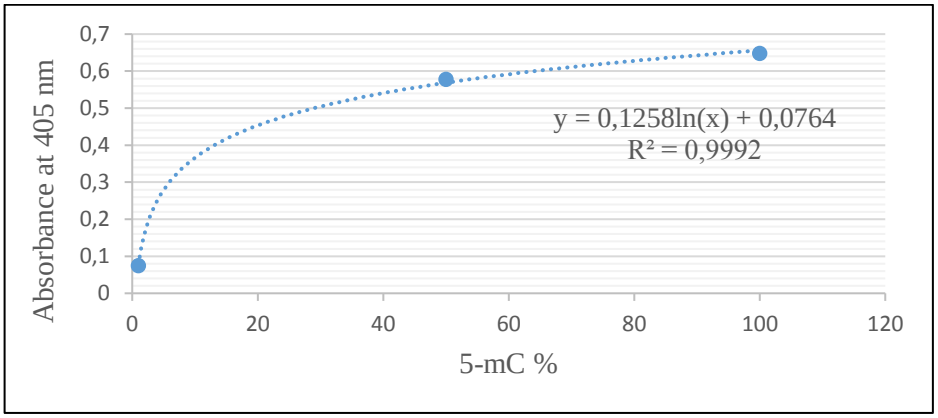

Figure 4.19. Standard Curve of 5-mC Global Methylation

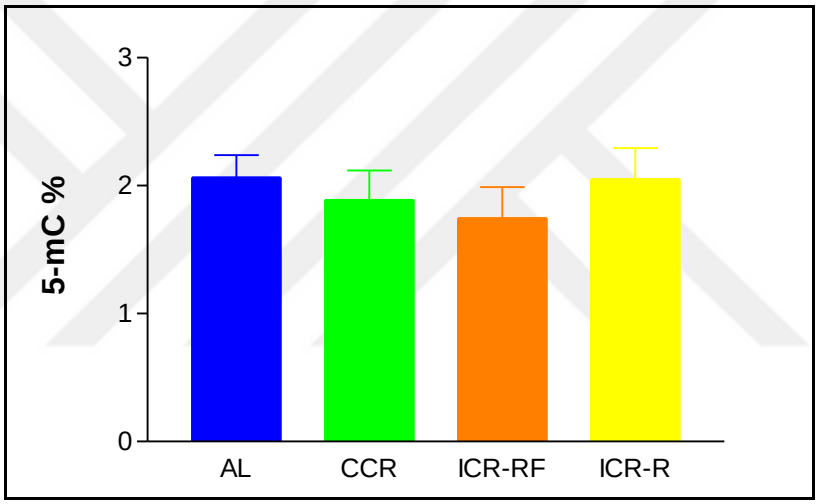

Figure 4.20. Global methylation levels in liver samples of MMTV-TGF-α breast cancer mouse model at week 81/82 "n" value represents samples taken from different animals (n = 6-11).

Furthermore, in order to analzye the aging effects on global methylation levels, each group was compared with a baseline (week 10) methylation levels. The difference between dietary groups was not significant (Figure 4.22).

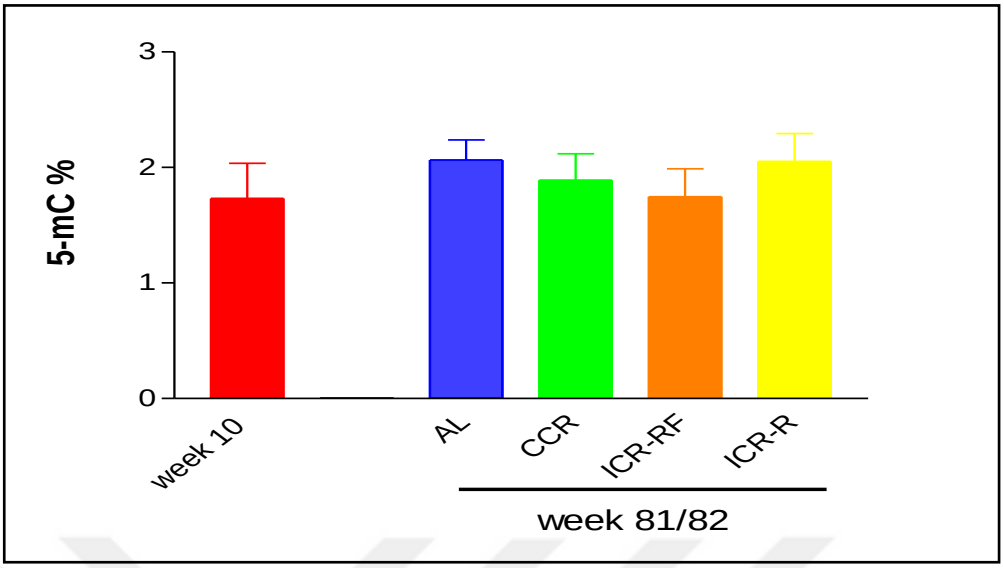

Figure 4.21. Changing in global methylation levels of MMTV-TGF-α breast cancer mouse model with aging "n" value represents samples taken from different animals (n = 6-11). x

5. DISCUSSION AND CONCLUSION

5.1. DISCUSSION

In the present study, by using MMTV-TGF-α positive mice, we studied the alterations in the methylation levels of *AdipoR1* and *LEPROT* genes which play significant roles in adipocytokine signaling under the effects of two different types of calorie restriction (Chronic Calorie Restriction and Intermittent Calorie Restriction) from week 10 until week 82 of mouse age. BW gain of mice in CCR group was significantly lower than the AL or ICR group. As predicted, mice in ICR group lost significant amount of BW during restricted periods of the study and gained back BW during re-feeding period throughout the study. These results were similar to the previous studies [21, 23, 131-133].

In the present study, CCR group was more protective than rest of the groups since CCR group had lower MT incidence rates compared to either AL or ICR group alone. In addition, there was no difference between AL and ICR group in terms of MT incidence rates. Although, our findings supports the beneficial effects of CR for MT development, it is different than the previous ones which report better protective role of ICR compared to CCR feeding for the MT development in mouse models [19, 21, 134]. This difference could be because of the application of the different style of ICR feeding regiments. In previous studies mice were applied to 25 per cent of CR for two weeks period in a month while mice in the current study were applied to 60 per cent CR for one week in a month in a cycle until they are sacrificed. In addition, in the present study 15 per cent CR was applied to CCR group while 25 per cent of CR was applied to CCR group in previous studies [22, 23, 56, 131]. On the other hand, similar to current study results, there are studies which reported similar MT incidence rates between AL and ICR group of mice [135-137]. For example, in previous studies MT incidence rates of AL and ICR groups were 50 per cent and 60 per cent respectively when four weeks of CR followed by an extended (12 weeks) period of *ad-libitum* feeding applied [135, 136].

Analyzing the effects of different types of CR on the methylation levels of *AdipoR1* and *LEPROT* genes at different time points throughout the life cycle, from week 10 until week 82 of mouse age was the purpose of the current study. Thus, the potential roles of methylation

of these two genes in the preventive effects of CR in MT development were clarified. The reasons these two genes chosen were because of their roles in the signaling pathways of adiponectin and leptin adipokines which were shown to play roles in MT development and obesity [19, 23, 25]. To our knowledge, the current study is the first study to report the effects of two different types of long term CR (CCR vs ICR) on the methylation levels of *AdipoR1* and *LEPROT* genes using mouse model.

The difference among dietary groups was not significant for the methylation level of *AdipoR1* gene at week 49/50. But, at week 81/82 when most of the MTs were developed, CCR group had two to four folds higher methylation levels of *AdipoR1* gene compared to the ICR and AL groups. This result is not unexpected and consistent with MT development incidence rate which was lower in CCR group. Although there are no similar previous studies to compare with our current findings, there are some studies report about the effects of different conditions or factors on the methylation levels of adiponectin and leptin signaling related genes [138-140]. For example, when the pregnant mothers were subjected to high fat diet containing 60 kcal per cent fat during late gestation period in mouse model for 20 weeks, the methylation levels of adiponectin gene and leptin receptor gene were increased while the methylation of leptin gene was decreased in the visceral fat tissue of the offspring [139]. In the same study, methylation levels of leptin and adiponectin related genes in liver and skeletal muscles were not changed. In another study, methylation levels of adiponectin itself was increased in adipocyte tissue of mice fed with 60 per cent high fat diet for 20 weeks [140]. In addition, higher methylation levels of adiponectin gene in DNA isolated from either blood or adipose tissue of human with high BMI was also reported [138]. In another human study, 36 hours of fasting increased methylation levels of promoter region of adiponectin gene extracted from subcutaneous adipose tissue of young men [141]. In addition, a different study conducted by Jiao *et al*. reported decrease in the methylation levels of adiponectin gene in visceral fat pad tissue of litters of mice fed with high fat diet during gestation and lactation [142]. Furthermore, using MS-PCR method no methylation levels of *AdipoR1* or *AdipoR2* genes extracted from the liver tissues of rats fed with and without the choline were observed [143]. Although these studies have stated the different effects of HFD on adiponectin or adiponectin receptor genes, all of them studied the short term (up to 20 weeks) dietary effects on methylation levels. However, in the present study, both the midterm (50 weeks) and long term (82 weeks) effects of two distinct types of CR on

methylation levels of genes related with adiponectin and leptin signaling pathways in transgenic mouse mammary tumor model were reported.

Previous studies reported higher serum adiponectin levels in CR groups which had lower BW and MT incidence rates compared to the AL group [19, 55]. Protective roles of adiponectin in MT development has been reported in recent studies. In this process, roles of adiponectin receptors, AdipoR1 and AdipoR2, have also been reported. Therefore, methylation levels of *AdipoR1* may play role in MT development by regulating *AdipoR1* gene expression levels and serum adiponectin levels. Unfortunately, adiponectin and leptin serum levels could not be measured in the current study because there was not enough amount of serum samples to be used since they were used in other studies previously.

In the present study, pyrosequencing method was used to measure methylation levels. The major advantage of pyrosequencing technique is to analyze not only global but also individual analysis of CpG island methylation levels. When specific CpG islands were investigated for the methylation levels of *AdipoR1* gene, in CCR group, methylation levels of CpG2 and CpG3 islands were significantly higher than that in AL group. This results indicate that methylation levels of CpG2 and CpG3 may play more significant roles in the effects of CR in metabolic or pathophysiological activities. In an another note, the methylation levels may seem to be lower in the present study. However, this is not unusual, since there are other studies which also report similar methylation levels of variety of genes including leptin gene in mice [144-146]. In these studies average methylation levels of promoter regions of genes of interests were ranged between 1-3 per cent [145, 147].

One of the other signaling pathways has been worked in the current study is leptin signaling pathways. Leptin is an adipokine that regulate energy balance and food intake [60]. The metabolic functions of leptin and its receptors (ObRs) are regulated by a small transcription factor called LEPROT. Recent studies claimed that silencing of *LEPROT* gene leads to an increase in leptin signaling. For this reason, regulators of leptin receptors become more convenient target to analyze for better understanding of leptin and its signaling associated proteins including its receptors [72]. In our study, the methylation levels of *LEPROT* gene were similar in all groups at week 49/50, however, methylation levels of CCR group was at least two fold lower than the other groups at week 81/82, although this difference was not statistically significant. This could be due to lower “n” values. More samples could not be analyzed because of financial restriction of pyrosequencing method. Lower methylation

levels of *LEPROT* in CCR group fits with the previous findings since proliferative effect of leptin in mammary tumor development has been shown in various studies both in-vitro and in-vivo [19, 55, 68]. Lower methylation of *LEPROT* increases *LEPROT* gene activation which inhibits leptin receptor activation. Therefore, MT growth rate could be slower in CCR group compared to the others due to lower activation of leptin signaling. Studies reported contradict 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 with HFD, some reported otherwise [146, 148-150]. In similar human studies, Houde *et al*. reported lower leptin methylation levels in blood and adipose tissue of people with high BMI [138]. In addition, lower methylation levels of leptin gene promoter region in human with high BMI was reported after 8 weeks of 30 per cent energy-restricted low calorie diet [151]. This results also supports our current findings.

When CpG islands of *LEPROT* gene in the promoter region were analyzed individually, methylation levels of CpG1 island showed significant difference amongst the dietary groups. Methylation levels of CpG1 in CCR group was three to five fold lower than the rest of the dietary groups at week 81/82, nevertheless, for rest of the CpG islands, statistically significant difference was not observed among the dietary groups. This indicates that CpG1 island of *LEPROT* gene may play pivotal role in leptin signaling pathway and MT development directly.

In the present study, changes in the methylation levels of *AdipoR1* and *LEPROT* genes with aging were also studied. In all dietary groups aside from *LEPROT* in CCR group, the methylation levels were decreased from week 10 to week 49/50 and then drastically increased at week 81/82. These result indicates that methylation levels of these two genes changes with aging and are modulated by CR which is one of the most effective ways to deal with aging . Interestingly, methylation levels of *LEPROT* gene was reversed by CCR types of CR which also decreased MT incidence rate in the present study. Therefore, the methylation levels of these two genes might be considered as aging biomarkers and could be used as targets for dealing with aging . In this context, the changes in serum leptin levels with aging has been reported in previous studies. Since adiponectin and leptin are interrelated, the present study may show adiponectin signaling related genes may also play role in aging. However, there are not any studies specifically report roles of methylation levels of these two genes in aging specifically, although most studies report increased

methylation levels of leptin and/or adiponectin signaling related genes with aging [67, 140, 148-150].

5.2. CONCLUSION

Present study demonstrated methylation levels of *AdipoR1* and *LEPROT* genes which play important roles in adiponectin and leptin signaling are modified by CR (Figure 5.1). When two different types of CR protocols are compared, CCR has more significant effect on methylation levels of both genes. This may be the explanation why CCR group had less MT incidence rate than either AL or ICR groups. In this study, with the exception of *LEPROT* methylation level in CCR group, the methylation levels of either genes also showed similar trend in all dietary groups with aging. It decreased at week 49/50 and then drastically increased at week 81/82. Methylation levels of *LEPROT* gene in CCR group decreased with aging while it increased in other groups. These results indicate that methylation levels of *AdipoR1* and *LEPROT* may play vital roles in the protective roles of calorie restriction in MT development. Also, our results indicate methylation of these two genes may also play role in protective effect of CR in aging process.

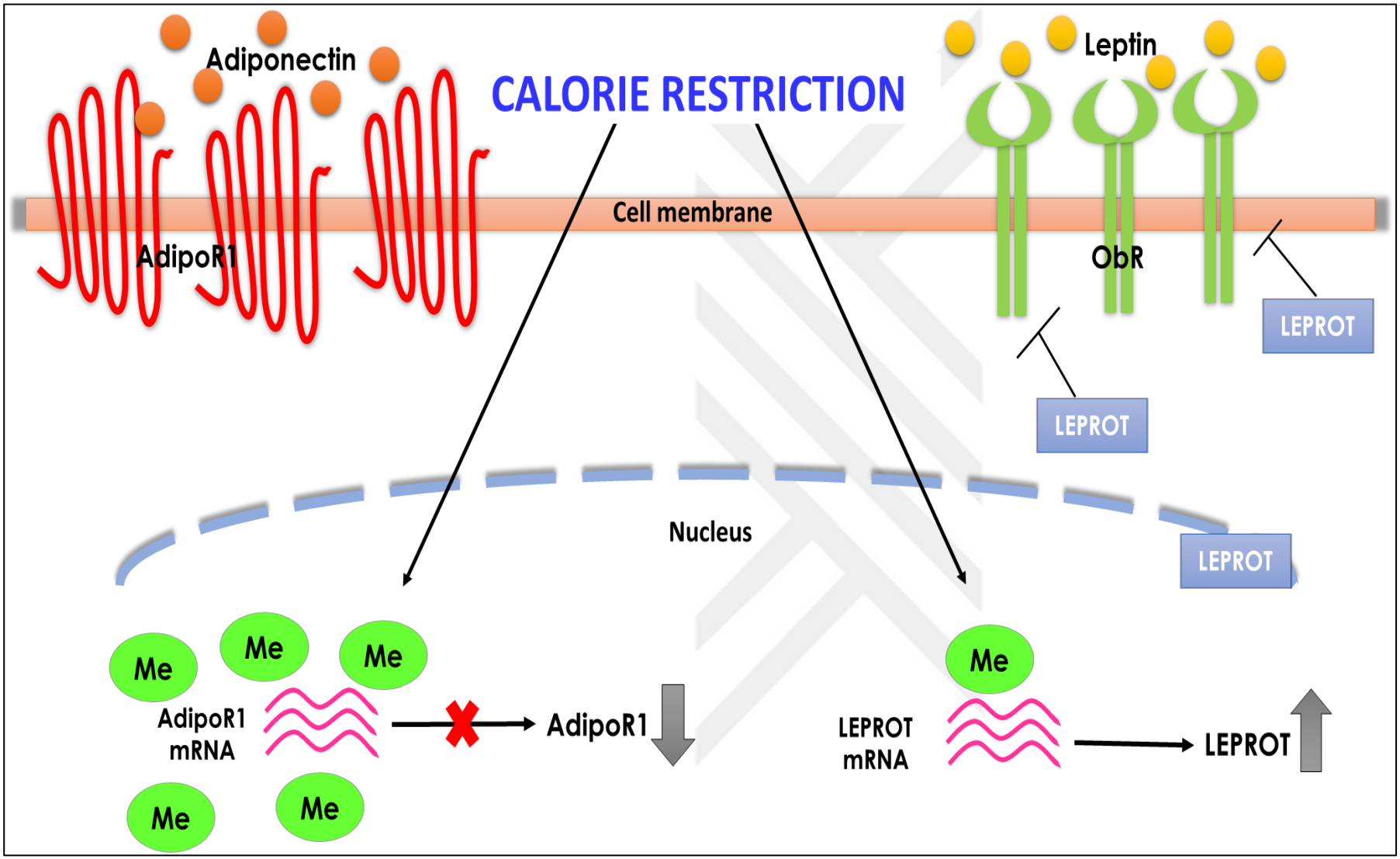

Figure 5.1. Calorie restriction regulates epigenetic pathways of *AdipoR1* and *LEPROT* genes

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