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RANKL IS A NEW EPIGENETIC BIOMARKER FOR THE VASOMOTOR SYMPTOM DURING MENOPAUSE

Kalkan R[1,2,*], Altarda M[1], Tosun O[3]

*Corresponding Author: Rasime Kalkan, Ph.D., Department of Medical Genetics, Faculty of Medicine, Near East University, Near East Boulevard, Nicosia, Turkish Republic of Northern Cyprus.
E-mail: kalkanr@yahoo. com

ABSTRACT

During menopausal transition, decreased level of estrogen brings a number of physiological problems and hormonal changes. In this study, promoter methylation of *RANKL* and *FSHR* genes were identified in 30 premenopausal and 35 postmenopausal women using methylation-specific high resolution melting (MS-HRM) analysis. The statistical analyses and their association with patient characteristics were performed by Pearson χ^2 and Fisher's exact test (p <0.05). The methylated *RANKL* gene was detected in 16 postmenopausal cases, and 12 (75.0%) of the *RANKL* methylated cases had hot flashes (p = 0.024). The methylated *FSHR* gene was detected in 18 postmenopausal cases, and 13 (75.0%) of the methylated cases had hot flashes (p = 0.028). In *vitro* studies demonstrated the association between *RANKL* expression, FSH level and hot flashes in the mouse. Although lack of epigenetic studies in this field proves our results crucial and therefore, our results showed magnitude of epigenetic profiles of Turkish Cypriot postmenopausal women. This was the first study which has investigated the *RANKL* and *FSHR* methylation and their relationship with hot flashes in postmenopausal women.

Keywords: Epigenetics; *FSHR*, *RANKL* genes; Hot flashes; Menopause.

INTRODUCTION

The first menstrual period and onset of menopause are the key milestones of female reproductive ageing [1].

Menopause occurs when the follicle pool in the ovaries has become exhausted and insufficient to maintain menstrual cycles [2]. Genetic and environmental factors are the major determinants of the ending and starting timing of these periods [3].

An increasing number of studies tried to identify the genetic background of reproductive ageing and disorders of reproduction [4-8]. Candidate-gene, genome-wide approach, linkage mapping and association studies tried to highlight genes that are related with the ending time of menstrual cycle. Genome-wide association studies (GWAS) described polymorphic loci in *GNRH1*, *HMCES*, *ZCCHC27*, *ZNF518A*, *NRB2*, *LY6G5C* and *BAG69* genes in European [7] and Japanese [9] populations, which was linked with menopause. Day *et al.* [10] reported the *PTPRD*, *PTPRF*, *PTPRJ*, *PTPRK*, *PTPRS* and *PTPRZ1* genes to be related to the age of menarche. Epigenetic age is another most popular topic in different types of diseases and recent studies identified statistically significant association with breast cancer susceptibility and increased epigenetic age [10]. Levine *et al.* [11] used different tissue types to be able to demonstrate the interaction between epigenetic aging and age at menopause. They showed increased epigenetic age in blood samples and this was associated with earlier menopause. The association between single nucleotide polymorphism (SNP), rs11668344 and age of menopause was also identified by Levine *et al.* [11].

An increasing number of studies tried to identify the genetic background of age of menopause and bone mass differences in menopause. The *CYP19A* [12], *HDC* [13], *ERα* [14-17], *FSHR* [18] gene polymorphisms are associated with hot flashes and bone mass during the menopause. In addition, until now, the interaction between epigenetics and hot flashes has not been reported in postmenopausal cases.

The *RANKL* gene is one of the members of the tumor necrosis factor (TNF) family [19,20] and *RANKL/RANK* genes are essential regulators of bone remodeling [21],

[1] Department of Medical Genetics, Faculty of Medicine, Near East University, Nicosia, Turkish Republic of Northern Cyprus

[2] Experimental Health Sciences Research Center (DESAM), Near East University, Nicosia, Turkish Republic of Northern Cyprus

[3] Department of Biostatistics, Faculty of Medicine, Near East University, Nicosia, Turkish Republic of Northern Cyprus

lymph node organogenesis [22] and formation of a lactating mammary gland [23]. These genes are also expressed in the main region of thermoregulation [22] and have an important role for thermoregulation [11]. Hanada *et al.* [17] tested central RANKL injection in mice and rats and this triggered a severe fever response.

The follicle-stimutating hormone (FSH) is another important hormone in female reproduction which acts *via* binding to the FSH receptor (FSHR). The interaction between *FSHR* gene mutations and reproduction in the female has been described by researchers [24-26]. Woad *et al.* [27] identified mutations that were associated with ovarian dysgenesis (NM_000145.3: c.566C>T) and FSHR function (NM_000145.3: c.1043C>G and NM_000145.3: c.1555C>A). Sun *et al.* [28], reported that FSH is able to directly stimulate osteoclast formation and metabolic activities *via* its receptor (FSHR). In *vitro* studies showed that *FSHR* null mice models demonstrate that FSH is required for postmenopausal bone loss [26-28]. Until now, genetic studies in menopause has been limited and researchers started to analyze a pool of different study populations under a large consortium to increase their power. These studies are based on the assumption of common variants or mutations underlying common bone mineral density, age at menarche and menopause. An increased number of studies in epigenetics helped to identify the genetic background of diseases, such as cancer and aging. In this study, instead of identification of structural genetic variants in menopause, we identified the epigenetic background of the hot flashes in menopause. Here, we reported methylation analysis of *FSHR* and *RANKL* genes in patients from Cyprus and identification of an epigenetic background of hot flashes during the menopause.

MATERIALS AND METHODS

The *FSHR* and *RANKL* promoter methylation was analyzed in 35 postmenopausal and 30 premenopausal women. The study was performed in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of the Near East University, Nicosia, Turkish Republic of Northern Cyprus. Written informed consent was obtained from all subjects participating in the study.

All participants were genetically unrelated postmenopausal females. Subjects with an unnatural menopause, took medications such as anxiolytics, antidepressants, exogenous hormone, women who have serious disease or mental retardation, smoking, alcohol usage and have a weight loss therapy, food allergies, heart disease history, insulin-dependent diabetes, diabetes mellitus type 2 (DMT2), kidney or liver disease, were excluded. Indi-

viduals matching the menopause criteria for >1 year, were recruited [29].

Genomic DNA was extracted from blood samples according to the AllPrep DNA/RNA/Protein isolation kit (Qiagen GmbH, Hilden, Germany), and its quantity was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at the Near East University Research Center of Experimental Health Sciences (DESAM), Nicosia, Turkish Republic of Northern Cyprus.

Determination of *FSHR* and *RANKL* Methylation Status. To determine the *RANKL* and *FSHR* methylation status, first bisulfite modification reaction was applied, and in this reaction, unmethylated cytosine residues were converted to uracil by bisulfite treatment of 1.3 μg DNA using the EpiTect Bisulfite Kit (Qiagen GmbH) according to the manufacturer's protocol. After conversion, DNA was eluted in buffer (Qiagen GmbH) to a final concentration of 30 ng/μL. Universal methylated and unmethylated DNA (EpiTect Control DNA Set; Qiagen GmbH) were used as methylated and unmethylated controls. We used Rotor Gene Q for methylation-sensitive high resolution melting (MS-HRM) analysis (Qiagen Ltd., Manchester, UK) to detect the methylation status of our samples. Primers were designed according to the EpiTect® HRM™ PCR (HRM-polymerase chain reaction) Handbook (Qiagen GmbH). We used comparable amounts of template genomic DNA for all samples resulting in cycle threshold (CT) values below 30 and differing by no more than three CT values as described previously [29].

Statistical Analyses. The statistical analyses and their associations with patient characteristics were performed by Pearson's χ^2 test and two-tailed Fisher's exact test. Calculations were performed using the Statistical Package for the Social Sciences SPSS® 16.0 software (SPSS Demo for Mac, Chicago, IL, USA), with a statistical significance of $p < 0.05$.

RESULTS

The mean (± SD) age of the 30 premenopause patients was 33.5 years (33.5 ± 6.9) and for the postmenopause patients 56.7 (56.7 ± 4.9).

The *RANKL* Methylation and Interactions with Hot Flashes. In the study group: *RANKL* promoter was methylated in 16 (45.7%) postmenopausal women and 19 (54.3%) were unmethylated (Table 1). In 12 (75.0%) of the 16 *RANKL* methylated postmenopausal cases had hot flashes. There was a statistically significant association identified between two groups ($p = 0.024$) (Table 2).

In the control group, 10 (33.3%) of the samples were methylated and 20 (66.7%) of the samples were unmethyl-

Table 1. Methylation status of *RANKL* and *FSHR* genes in pre- and postmenopausal women.

Genes	Postmenopause	Premenopause	Positive Hot Flashes History	Negative Hot Flashes History
RANKL (M) *n*	16 (45.7%)	10 (33.3%)	12 (75.0%)	4 (25.0%)
RANKL (U) *n*	19 (54.3%)	20 (66.7%)	7 (36.8%)	12 (63.2%)
FSHR (M) *n*	18 (51.4%)	20 (66.7%)	13 (72.2%)	5 (27.8%)
FSHR (U) *n*	17 (48.6%)	10 (33.3%)	6 (35.3%)	11 (64.7%)

M: methylated; U: unmethylated.

Table 2. Methylation status of *RANKL* and *FSHR* genes and postmenopausal women who have hot flashes.

Genes	Positive Hot Flashes History	Negative Hot Flashes History	*p* Value
RANKL (M) *n*	12 (75.0%)	4 (25.0%)	0.024
RANKL (U) *n*	7 (36.8%)	12 (63.2%)	
FSHR (M) *n*	13 (72.2%)	5 (27.8%)	0.028
FSHR (U) *n*	6 (35.3%)	11 (64.7%)	

M: methylated; U: unmethylated.

ated (Table 1). There was no statistically significant association between menopause and methylation of the *RANKL* promotor ($p > 0.05$) (Figure 1).
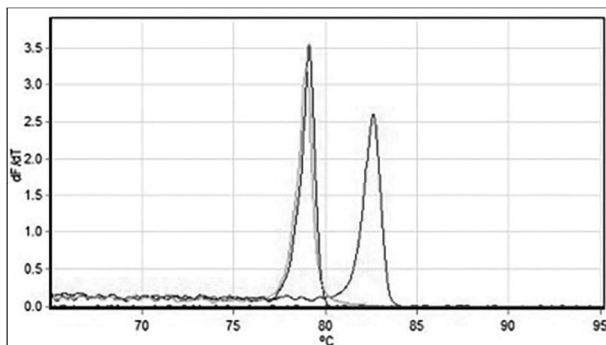

Figure 1. Methylated RANKL patient. The RANKL unmethylated control is purple, methylated control is red. Patient #50 was methylated and patient #52 was unmethylated.

The *FSHR* Methylation and Interactions with Hot Flashes. The *FSHR* gene was methylated in 18 postmenopausal cases and unmethylated in 17 postmenopausal cases. In the control group, *FSHR* genes were methylated in 20 cases and unmethylated in 10 cases. There was no statistically significant association between two group ($p > 0.05$) (Table 2).

Thirteen (75.0%) of the 18 *FSHR* methylated postmenopausal cases had hot flashes. Statistically significant association was identified between the two groups ($p = 0.028$) (Table 2).

DISCUSSION

Several studies focused on identification of interactions between methylation and cancer. Ambatipudi *et al.*

[2], worked with epigenetic ageing and they showed accelerated epigenetic age was associated with CpG island methylation and breast cancer susceptibility in postmenopausal women [2]. Epigenetic-based studies in postmenopausal women is limited and the largest part of the studies were related to mutational screening. To the best of our knowledge, this study is the first study which shows the epigenetic alterations in menopause and the epigenetic background of hot flashes in postmenopausal women.

Mutations of the *FSHR* gene analyzed in different groups, such as premature ovarian failure (POF) [30-37], low number of oocytes [36], age of menopause [37], infertility [38], migraine [39], bone mineral density and bone turnover [18]. Woad *et al.* [27] searched FSHR variants in exons 7 and 10 in patients who suffered with POF. They identified a novel heterozygous NM_000145.3:c.1411A>T variant in FSHR exon 10 in a family who have a history of POF and elevated FSH concentration [27]. The FSHR mutation and POF interaction has been studied by several researchers [30-34,40].

Rendlina *et al.* [34] found that the FSHR rs6166 polymorphism significantly influenced bone mineral density (BMD) in postmenopausal women. Cordts *et al.* [8] concluded that the NM_000145.3: c.919G>A polymorphism in the *FSHR* gene was associated with premature ovarian insufficiency (POI) development and could be used as a screening marker in patients with ovarian failure [8].

Our study is the first study based on current literature that has shown the interaction between FSHR promoter methylation and hot flashes in postmenopausal patients. The methylated *FSHR* gene detected in 18 postmenopausal cases and 13 (75.0%) of the methylated cases had hot flashes. There was statistically significant association between the two groups ($p = 0.028$). This was the first study

which showed the interactions between FSHR methylation and hot flashes in postmenopausal women.

The *RANKL* gene is important for bone homeostasis and related biological processes [41]. In fact, the *RANKL* gene has been linked as a candidate gene for osteoporosis susceptibility and also confirmed by GWAS, as a susceptibility locus regulating BMD [42]. Single nucleotide polymorphisms of the RANKL promoter showed association with BMD [41,43,44]. Mencej *et al.* [25] showed that promoter polymorphisms of the *RANKL* gene was related with elevated transcriptional activity of the gene and correlated with increased RANKL protein rate. This caused imbalances in bone metabolism and was related with low BMD [44]. Shang *et al.* [40] analyzed SNPs of the *RANK* and *RANKL* genes in a Chinese female population and showed that the RANKL polymorphisms related with BMD in the femoral neck in peri- and postmenopausal Chinese women [41]. Researchers tried to identify the importance of genetic variations in peri- and postmenopausal women and BMD. The importance of SNPs in BMD has been shown by different researchers [27,38,45,46].

Delgado-Calle *et al.* [12] showed that increased expression of RANKL during osteoporotic fractures but did not show statistically significant association between methylation and osteoporotic fractures. In *vitro* studies demonstrated the association between RANKL expression and thermoregulation during postmenopause and also homozygous NM_003701.3: c.508A>G mutation in the *RANKL* gene was associated with impaired fever response [47].

Hanada *et al.* [16] demonstrated that central RANKL injection in mice and rats triggered severe fever and they mapped RANK during the fever response on astrocytes, on the other hand, when they applied high doses of RANKL in the intraperitoneal this did not cause any changes in body temperature or in activity. They highlighted RANKL as an important fever inducer in the central nervous system [16]. Hanada *et al.* [16] concluded that RANKL/RANK regulates female body temperature and they also demonstrated that ovariectomy-induced changes in core body temperatures occurred in RANK knockout female mice. Hanada *et al.* [16,17] identified that genetical inactivation of RANK in the brain resulted in an altered physiological thermoregulation in female mice, which, at least in part, appears to be regulated by ovarian sex hormones.

In this study, we detected *RANKL* promotor methylation and hot flashes in 12 (75.0%) postmenopausal women ($p = 0.024$) (Table 2). Researchers highlighted that RANKL used a COX2-PGE(2)/EP3R pathway for thermo-regulation and induction of fever. We suggested that increased methylation of RANKL directly effects the expression on the gene and this causes abnormal fluctuation of temperature

on postmenopause patients. At the same time, researchers highlighted that deficiency of RANKL/RANK expression is related to osteoporosis and irregular resorption of bone. In accordance with the literature, we can conclude DNA methylation is another mechanism which decreased gene expression and increased irregular resorption of bone but this hypothesis also needs confirmation on bone cells. Here, consistent with the Hanada *et al.* [16,17] findings, we demonstrated significant association between the *RANKL* gene methylation and hot flashes in postmenopausal women. On the other hand, we have found significant differences in the gene methylation profiles of the postmenopausal and premenopausal women, including genes that their epigenetic changes have not yet been associated with postmenopausal women. Our findings may provide further insight into the process of postmenopausal changes and gene methylation status. This was the first study that investigated the epigenetic changes of *RANKL* and *FSHR* genes in postmenopause and their relation with hot flashes.

Conclusions. In conclusion, we found significant association between the *RANKL* and *FSHR* gene methylation and hot flashes in postmenopausal women. Future studies, in larger samples of postmenopausal women, should focus on the study of different gene methylations, and this will help to clarify potential effects of gene methylation in menopause.

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