Determination of KISS1, KISS1R and Kisspeptin in Fat Tissue of Normal Weight and Obese Humans and Correlations between Serum Kisspeptin and Leptin

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ABSTRACT

Kisspeptin, a neuropeptide mainly expressed in the hypothalamus, has a major role in reproductive regulation. Previous studies showed that Kiss1 mRNA expression is also found in adipose tissue in rats. This study aimed to determine KISS1, KISS1R, and leptin expression in fat tissues. Both KISS1 and KISS1R mRNA expressions were extremely low in human subcutaneous and visceral adipose tissues in real-time PCR analysis. Nested real-time PCR was done for both KISS1 and KISS1R primers showing that the expressions of KISS1 and KISS1R mRNA were highly determined in visceral, but not in subcutaneous adipose tissues. Serum kisspeptin levels were not different between obese and normal weight subjects and were positively correlated with serum leptin ($R=0.381$, $p<0.05$). In conclusion, KISS1 and KISS1R expressions were detected in visceral adipose tissue.

Keywords: KISS1, KISS1R, kisspeptin, leptin, fat tissue

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INTRODUCTION

The KISS1 gene encodes a 145 amino acid neuropeptide, kisspeptin, which has a major role in reproductive regulation[1]. The peptide is cleaved into an unstable 54 amino acid peptide[2], the most bioactive form in vivo[3] with a short half-life[4] and is degraded into the shorter peptides, including kisspeptin-14, kisspeptin-13, and kisspeptin-10[2]. Kisspeptin binds to kisspeptin receptor, KISS1R/Kiss1R (for the human/ non-human genes, respectively), which is also called G protein-coupled receptor-54 (GPR54). Kiss1 gene expression is found in the arcuate nucleus (ARC) and preoptic area (POA) of the hypothalamus and is activated directly by leptin[5] as Kiss1 neurons in these areas express leptin receptors[6]. Kisspeptin can directly activate the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons expressing GPR54 located at the POA[7]. Moreover, Kiss1 and GPR54 are also expressed in peripheral tissues, including the pituitary gland, ovary, placenta, pancreas, smooth muscle of human coronary artery, aorta, and umbilical vein[1]. Interestingly, gene expression profiles of several hormones expressed in human fat are also expressed in the hypothalamus, including NPY, corticotropin-releasing factor (CRF), melanin-concentrating hormone (MCH), and orexin[8]. Conversely, some adipokines including resistin and leptin are also expressed in the brain, including the cerebral cortex, and hypothalamus[9], suggestive of an association among central and peripheral regulation of energy metabolism and reproduction. This study aimed to determine KISS1 and KISS1R mRNA in fat tissues. Moreover, serum kisspeptin and leptin were also determined. The results might reveal associations between leptin and kisspeptin which are involved in energy metabolism and reproductive regulation.

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MATERIALS AND METHODS

Subjects

The study protocol was approved by the Siriraj Institutional Review Board (Si.490/2011) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The patients gave informed consent prior to the study. Totally, 35 Thai female patients who underwent abdominal surgery were recruited who were obese (n=20) (BMI >25 kg/m^2), normal weight (n=11) (BMI 18.5-22.9 kg/m^2), overweight (n=2) (BMI 23-24.9 kg/m^2), and lean (n=2) (BMI <18.5 kg/m^2) for clinical, anthropometric and blood data collection. However, fat tissue could be obtained from only 30 patients including 17 from obese, 9 from lean, 2 from overweight, and 2 from lean group. Comparisons between obese and normal weight groups were made and subjects in the overweight and lean groups were included for correlation analysis. Subjects with any of the following; endocrine therapy (e.g. steroids, hormone replacement therapy, thyroxine), pregnancy, lactation, traumatic operation, malignancy diseases, operation related to endocrine diseases, severe abdominal inflammation, or menopause were excluded. In this study, male subjects could not be recruited because most male patients who underwent open abdominal surgery were cancer or emergency operations. The phase of menstrual cycle of each female subject recruited in this study could not be controlled because most of the subjects had myoma uteri displayed with irregular menstruation.

Demographic details and anthropometric measurements

Demographic details of patients were collected including age, BW, BMI, waist circumference (WC), hip circumference (HC), and waist to hip circumference ratio (WHR). In standing position, WC was measured at the level of the umbilicus with silent breathing and HC was measured at the inter-trochanteric girth according to the WHO guideline[10]. WHR is calculated by WC divided by HC.

Tissue and blood collection

Blood samples were collected in the fasting state before the operation. Four to five pieces of 0.5 cm of each type of adipose tissues, which were abdominal subcutaneous and visceral adipose tissues, were collected. Adipose tissues were immediately snap-frozen in liquid nitrogen and stored at -70˚C.

Analysis of gene expression of KISS1, KISS1R, and leptin in adipose tissues

The total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. About 1 µg of RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-RAD, Hercules, California, USA). Low density lipoprotein receptor-related protein 10 (LRP-10) was used as a reference gene, because it is the most stably expressed gene in human adipose tissue[11]. The real-time polymerase chain reaction (real-time PCR) primer sequences were obtained from previously published data for KISS1 and KISS1R in renal cell carcinoma[12]. However, KISS1 and KISS1R expressions were extremely low in human adipose tissues compared to high expression of both KISS1 and KISS1R in placenta, which was used as a positive control. As a result, nested real-time PCR was done to further investigate the expression in fat tissues. Leptin, inner KISS1 and inner KISS1R primers were designed by the authors using published nucleotide sequences from PubMed database. The primers were designed with the exon to exon sequences to confirm their specificity to mRNA and were blasted to all species sequences to prove their specificity to the gene of interest. The real-time PCR and nested real-time PCR primer sequences have been shown in Table 1. In order to quantify KISS1, KISS1R, leptin, and LRP-10 mRNA expressions, the real-time PCR reactions were carried out using the reagents and protocol contained in the VeriQuest SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, California, USA). The annealing temperature was 62ºC for KISS1 and KISS1R, 58ºC for leptin, and 57ºC for LRP-10. For nested real-time PCR, the inner primers for KISS1 and KISS1R and 2 µl of the product from the previous real-time PCR reaction were used and the annealing temperature was 63ºC for KISS1 and 62ºC for KISS1R. For every real-time PCR reaction, no template control (NTC) was performed as a negative control. The size of real-time PCR products was determined by electrophoresis. The $2^{-\Delta CT}$ method was applied as a comparative method of quantification.

TABLE 1. The real-time PCR, nested real-time PCR primer sequences and product size of each gene.

Genes	Sequence	Product size (base pair)
Outer KISS1-forward	5'-CACTTTGGGGAGCCATTAGA-3'	294
Outer KISS1-reverse	5'-CCAGTTGTAGTTCGGCAGGT-3'	
Inner KISS1-forward	5'-AATTCTAGACCCACAGGCCA-3'	249
Inner KISS1-reverse	5'-GTAGTTCGGCAGGTCCTTC-3'	
Outer KISS1R-forward	5'-GGACCGTGACCAACTTCTACA-3'	290
Outer KISS1R-reverse	5'-AGAGCCTACCCAGATGCTGA-3'	
Inner KISS1R-forward	5'-AACTACATCCAGCAGGTCTC-3'	149
Inner KISS1R-reverse	5'-ACCCAGATGCTGAGGCTGA-3'	
Leptin-forward	5'-CAATGACATTTCACACACGCAGTC-3'	312
Leptin-reverse	5'-GCCACCACCTCTGTGGAGTAG-3'	
LRP-10-forward	5'-GATGGAGGCTGAGATTGTGCA-3'	169
LRP-10-reverse	5'- TGGAGTCATATCCTGGCGTAAG-3'	

Immunofluorescent staining

Adipose tissue was fixed in 4% paraformadehyde overnight, snap-frozen with liquid nitrogen, and kept at -70°C. The tissue was dehydrated, paraffin embedded, sectioned at 20 µm thickness, and mounted on hydrophilic surface Twin-Mark microscope slides (Citotest Labware Manufacturing, Nanjing, Jiangsu, China). Immunofluorescent detection of kisspeptin was performed according to the published paraffin protocol from Abcam®. The primary antibody was 1:100 dilution of mouse anti-human KISS1 monoclonal antibody H00003814-M05 (Abnova, Neihu District, Taipei City, 114 Taiwan) and the secondary antibody was 1:20,000 dilution of Goat Anti-Mouse lgG (H&L) conjugated with fluorescein isothiocyanate (FITC) (Abnova, Neihu District, Taipei City, 114 Taiwan). BisBenzimide Hoechst 33258 (Sigma, St Louis, MO) was used for nuclear counterstain at a final concentration of 2.5 µg/ml. Negative controls were performed by omission of primary antibody. Human placenta was used as a positive control for kisspeptin staining.

Analysis of serum kisspeptin and leptin levels

Serum kisspeptin levels were measured by a commercial enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals Inc., Burlingame, California, USA) and serum leptin levels were measured by a commercial enzyme-linked immunosorbent assay (ELISA) Kit (Phoenix Pharmaceuticals Inc., Burlingame, California, USA). The ranges of kisspeptin and leptin detection were 0-100 ng/ml and 0.31-20 ng/ml, respectively, and the minimum detectable concentrations were 0.06 ng/ml and 0.312 ng/ml, respectively. The absorbance O.D. was read at 450 nm by Synergy HT Multi-Detection Microplate Readers (BioTek Instument, Inc., Winooski, VT, U.S.). Intra- assay coefficients of variances were 5.867% and 8.955%, respectively.

Statistics

Data were presented as mean±S.E.M. The Kolomonov-Smirnov test was performed to test normality. Comparisons between obese and normal weight groups were performed by unpaired T-Test and comparisons between subcutaneous and visceral adipose tissues were made by paired T-Test because all data were normally distributed. Correlation coefficients were calculated using a 2-tailed Pearson product-moment correlation method. A p value less than 0.05 was considered as statistical significance.

RESULTS

Comparisons between obese and normal weight subjects

The mean ratio of age and BMI of obese/normal weight subjects, mean difference of BMI between obese and normal weight subjects, WC, HC, WHR, serum leptin, and serum kisspeptin were compared between obese and normal weight subjects as shown in Table 2.

KISS1, KISS1R and leptin expression in adipose tissues

In real-time PCR analysis, both KISS1 (Fig 1A, 2A & B) and KISS1R (Fig 1B, 2E & F) mRNA expressions were extremely low in human subcutaneous and visceral fat, but were highly expressed in placenta (Fig 1A & B, 2A, B, E & F). The dissociation curve showed high amplitude in placenta and very low levels in fat tissue for KISS1 (Fig 2B) and KISS1R (Fig 2F). Nested real-time PCR showed that KISS1 (Fig 1A, 2C & D) and KISS1R (Fig 1B, 2G & H) expressions were highly determined in placenta and visceral fat (Fig 2C), but not in subcutaneous fat. The dissociation curve showed high amplitude in both placenta and visceral fat for KISS1 (Fig 2D) and KISS1R (Fig 2H). KISS1 and KISS1R mRNA in subcutaneous and visceral fat was not significantly different between obese and normal weight subjects. Kisspeptin protein expression, shown in green, was strongly detected in cytoplasm of human placenta tissues (Fig 3A), but was not detected in fat (Fig 3C). In order to confirm the quality of the fat tissues and the experiments, leptin mRNA in subcutaneous and visceral fat tissues was determined as shown in Fig 1C.

Correlations between 2 factors

Serum kisspeptin levels were positively correlated with serum leptin levels ($R=0.381$, $p<0.05$).

DISCUSSION

This study revealed that KISS1 and KISS1R expressions were extremely low in human fat tissues compared to high expression in placenta. Consequently, nested real-time PCR was done. KISS1 and KISS1R were highly detectable in visceral fat tissues and placenta, but not in subcutaneous adipose tissue with comparable detection

TABLE 2. Parameters of the subjects compared between obese and normal weight subjects.

Parameters	Obese (n=20)	Normal weight (n=11)
Mean ratio of age of obese/normal weight subjects	1.08	1
Mean ratio of BMI of obese/normal weight subjects	1.45***	1
WC, cm (mean±S.E.M.)	93.07±2.88***	74.35±2.44
HC, cm (mean±S.E.M.)	105.60±1.91***	90.27±1.79
WHR (mean±S.E.M.)	0.88±0.15*	0.82±0.02
Serum leptin, ng/ml (mean±S.E.M.)	59.99±6.99***	16.21±3.51
Serum kisspeptin, ng/ml (mean±S.E.M.)	1.15±0.03	1.06±0.07

Values are expressed as mean±S.E.M.
***p<0.001, **p<0.01,*p<0.05 compared with normal weight

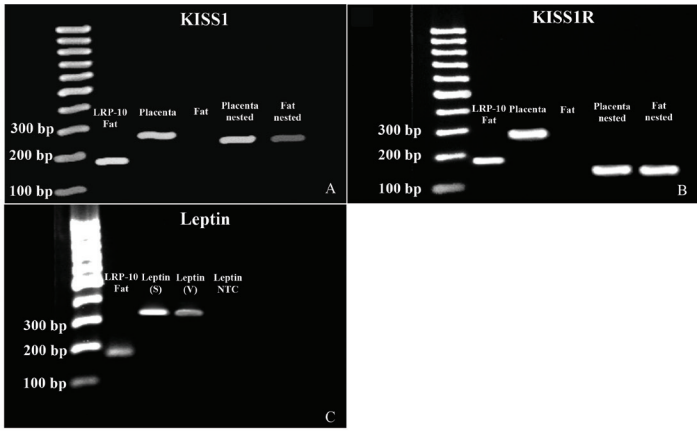

Fig 1. Gel electrophoresis reveals expression of KISS1, KISS1R and leptin mRNA .

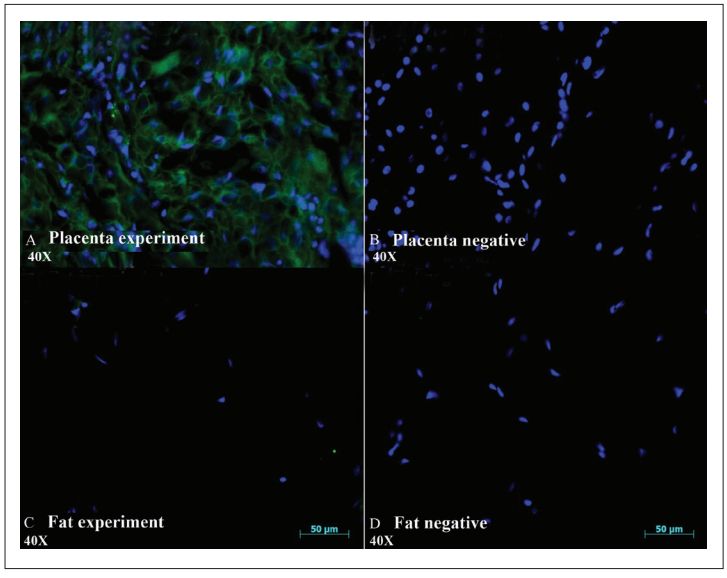

Fig 3. Immunofluorescent staining reveals kisspeptin protein expression (shown in green) and Hoechst 33258 for nuclear staining (shown in blue). Panel A&B present kisspeptin protein expression in placenta for positive and negative control, respectively, and Panel C&D present kisspeptin protein expression in fat tissue for experiment and negative control, respectively.

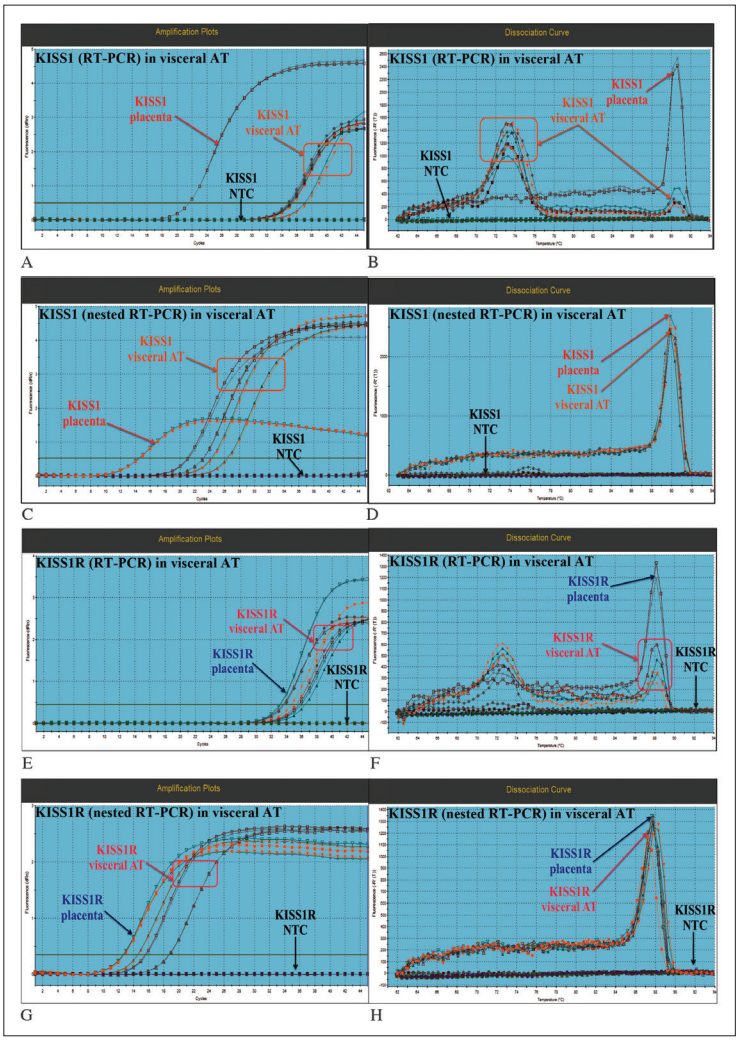

Fig 2. The amplification plots and dissociation curve of KISS1&KISS1R. Panel A&E: the amplification plots of KISS1&KISS1R, respectively, in real-time PCR showing that Ct was 22 and 33, respectively, in placenta and was 34-37 and 35-37, respectively, in visceral fat tissue. Panel C&G: the amplification plots of KISS1&KISS1R, respectively, in nested real-time PCR showing that Ct was 13 and 12, respectively, in placenta and was 20-25 and 14-18, respectively, in visceral fat tissue, Panel B&F: the dissociation curve of KISS1&KISS1R, respectively, in real-time PCR, Panel D&H: the dissociation curve of KISS1&KISS1R, respectively, in nested real-time PCR.

between obese and normal weight subjects. Immunohistochemistry staining was done to confirm kisspeptin protein expression in human fat tissue showing that kisspeptin protein was not determined in fat tissue. All of these results suggested that fat tissues might synthesize very small amounts of kisspeptin and kisspeptin might not have clinical importance on adipose tissue regulation. Our results were consistent with a previous study in humans[13] showing that KISS1 was highly detected in placenta and was almost undetectable in adipose tissue. However, recent studies in rats showed that Kiss1 mRNA expression was found in adipose tissues and Kiss1 mRNA was found in basal hypothalamus compared to fat and pituitary gland (100:8:1)[14]. The discrepancy of results might be due to the fact that the size of human adipocytes is about 30-40% bigger than that of mice and rats. Thus, RNA content/fat volume might be higher in mice and rats than in humans. Consequently, gene expression could be detected at higher levels in rat or mice adipose tissue[15]. Our result for KISS1R mRNA was consistent with a previous study showing low expression in human adipose tissues and much higher expression in pituitary gland, placenta, brain, pancreas, and lymphocyte[13]. Interestingly, both KISS1 and KISS1R mRNA expressions were detected in visceral adipose tissue, but not in subcutaneous adipose tissue. Generally, there are differences between subcutaneous and visceral fat depots including anatomical location, size, terms of adipokine expressions, and physiological and metabolic differences[16-19]. Interestingly, serum kisspeptin and leptin levels were positively correlated a result from a previous study in control Tanner I girls, prepubertal obese girls and girls, with idiopathic central precocious puberty (CPP)[20]. In the hypothalamus, leptin activates KISS1 neurons[5] as Kiss1 neurons in these areas express leptin receptors[6], although, recent data showed that not all kisspeptin neurons in the hypothalamus are under

control of leptin[21]. In peripheral tissue, cells expressing the KISS1 gene express leptin receptor, including pituitary[22], syncytiotrophoblast,[23] and ovary[24] in humans and other species, suggesting that leptin might affect KISS1 gene expression and kisspeptin synthesis. However, the effect of leptin on peripheral kisspeptin is still unknown. Further studies are necessary to confirm the role of leptin on kisspeptin secretion, especially on peripheral tissue.

In conclusion, this study revealed that KISS1 and KISS1R were detected in human visceral fat tissue by nested real-time PCR. Serum kisspeptin was positively correlated with leptin. There might be a relationship between peripheral kisspeptin and a key hormone produced in obesity. The role of peripheral kisspeptin needs to be studied further to confirm the linkage between reproduction and metabolic status.

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