Original Article

MicroRNA-125a-3p expression in abdominal adipose tissues is associated with insulin signalling gene expressions in morbid obesity: observations in Taiwanese

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Background: Micro (mi) RNAs have been found to play an important role in the regulation of adipogenesis and insulin sensitivity. However, associations between miRNA and insulin signalling-related gene expressions in abdominal adipose tissues in obese subjects remain unclear. Methods: We used a microarray platform to screen miRNA expressions in abdominal adipose tissues between genders in severely obese subjects and found that the top-ranking miRNA in abdominal omental adipose tissues was miRNA-125a-3p. MicroR-125a-3p and insulin signalling-related gene expressions in abdominal omental adipose tissues of all subjects (11 men and 10 women) were subsequently quantified by a real-time PCR. Also, associations of miR-125a-3p with insulin signalling-related gene expression and biochemical markers in obese subjects were analyzed by a linear regression analysis. Results: miR-125a-3p expressed by abdominal omental adipose tissues was much higher in obese men than women. No gender difference was observed in abdominal subcutaneous adipose tissues. Concomitant with high miR-125a-3p, c-Jun N-terminal kinase gene expression was also higher, whereas insulin receptor was lower in men than women. There were negative associations of miR-125a-3p with the insulin receptor and phosphatidylinositol 3-kinase expressions. Fasting plasma glucose and cholesterol levels were positively associated with miR-125a-3p expression. These associations were obvious in obese men but not women. Conclusion: Our results support the involvement of miR-125a-3p in regulating the insulin signalling pathway and imply that increased miR-125a-3p expression in omental adipose tissues may be a characteristic feature of insulin resistance in obese men.

Key Words: morbid obesity, abdominal adipose tissue, miR-125a-3p, insulin signalling pathway, insulin resistant

INTRODUCTION

MicroRNAs (miRNAs) are small, noncoding RNAs of approximately 21-25 nucleotides in length. MiRNAs are thought to be involved in regulating various normal biological processes because miRNAs recognize target mRNAs through partial complementarity to specific sequences within the mRNAs and regulate posttranscriptional gene expressions.[1-3] MiRNAs are predicted to act on multiple targets and quantitatively alter expressions of their target genes and proteins. Although changes in miRNA expression levels have been found to be correlated with a variety of diseases, the precise nature and downstream effects of such alterations are largely unknown.

Obesity is a worldwide epidemic. Obesity and the associated metabolic syndrome are major public health issues. Obese individuals are at increased risk of illnesses seen less often than those with normal body mass indices (BMI). Obesity in both men and women is associated with various aberrations including glucose intolerance, insulin resistance, an increased propensity to diabetes, and lipid profile abnormalities.[4] There is growing evidence of the important role of miRNAs in regulating the adipogenesis pathway, insulin resistance, and inflammation.[5] However, associations between miRNA and insulin signalling-related gene expressions in abdominal adipose tissues in obese subjects remain unclear. Differences in

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miRNAs expressed by intra-abdominal omental adipose tissues between genders have not been investigated.

In this study, we used a microarray platform to screen miRNA expressions in abdominal adipose tissues between genders in severely obese subjects. We found that the top-ranking miRNA in abdominal omental adipose tissue was miRNA-125a-3p. MiR-125a is one of the many miRNAs that remain to be fully characterized. Pre-miR-125a generates two mature miRs: miR-125a-3p and miR-125a-5p.[6] A previous study found that miR-125a is overexpressed in insulin target tissues in a rat model of type 2 diabetes mellitus (T2DM).[7] Those researchers predicted that target genes of miR-125a are involved in lipid metabolism and mitogen-activated protein kinase (MAPK) signalling pathways. At present, very little is known about the regulation of glucose homeostasis by miR-125a, especially miR-125a-3p, between genders in obese subjects. Therefore, in this study, we compared differences in abdominal adipose tissue miR-125a-3p expressions in morbid obese men and women to elucidate the possible regulatory role of miR-125a-3p in insulin signalling-related gene expression levels and its association with biochemical markers.

METHODS

Subjects

Patients aged 20-50 years who were scheduled for weight -loss surgery and met the criteria recommended by the US National Institute of Health (NIH) of a BMI of ≥40 kg/m^2, or a BMI of ≥35 kg/m^2 with obesity-related morbidity, were enrolled in this study at Taipei Medical University Hospital (Taipei, Taiwan) in March to June, 2012. All patients signed informed consent in order to take part in the project. This study was approved by the Research Ethics Committee of Taipei Medical University Hospital. Twenty-one patients (11 men and 10 women) who underwent sleeve gastrectomy were included. Patients were weighed and measured under standardized conditions, i.e., 2 h fasting and no physical exercise 12 h prior to the measurement. All subjects had a stable weight with no fluctuations of more than 2% of the body weight for at least 3 months before surgery. The body weight and height were measured by the same person using an electronic balance with a stadiometer (with a maximum weight of 250 kg) and were recorded to the nearest 0.1 kg and 0.1 cm, respectively. A body-composition analysis was performed before surgery by the multiple-frequency bioelectrical impedance method using InBody-230 (Biospace, Seoul, Korea) to study the muscle mass (MM) and fat mass (FM). A bioelectrical impedance method was found to have high relative agreement with dual-energy x-ray absorptiometry in measuring the body composition of obese subjects.[8] The waist circumference was defined as the abdominal circumference above the iliac crest, the hip circumference was measured in a horizontal plane at the level of the maximal extension of the buttocks, and the waist-hip ratio (WHR) was calculated. Paired samples of abdominal subcutaneous and intra-abdominal omental adipose tissues were obtained from all patients, and immediately frozen in liquid nitrogen.

Measurement of plasma parameters

Routine clinical chemistry was measured before the surgery. Blood samples were obtained after 8 h of fasting overnight for the analysis of plasma glucose (AC), cholesterol, triglyceride (TG), and high density lipoprotein-cholesterol (HDL-C). The biochemical parameters were measured using an automatic analyzer (Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Hemoglobin (Hb) A1C was measured with an HLC-723 G7 analyzer (Tosoh, Tokyo, Japan). There were 11 patients with normal glucose levels and 10 subjects diagnosed as T2DM with 5 men and 5 women. Three men and 2 women were treated with hypoglycemic drugs (4 patients used metformin and 1 patient a sulfonylurea), the other patients were instructed in blood glucose control by diet.

Total RNA isolation

Total RNA was extracted from adipose tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA concentration and purity were checked by examining the OD260/OD280 (>1.8) and OD260/OD230 (>1.8) ratios, and the RNA yield and quality were accessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Oligonucleotide DNA microarray (Human miRNA OneArray®)

The human miRNA OneArray® vers. 4 (Phalanx Biotech Group, Hsin Chu, Taiwan) contains triplicate 1884 unique miRNA probes from humans and primates (miRBase Release 18.0), and 144 experimental control probes. A detailed description of the gene array list is available from http://www.phalanx.com.tw/tech_support/gene_lists_miRNA.html.

Microarray analysis

Three equal-amount RNA samples from randomized selected men or women extracted from abdominal omental or subcutaneous tissues were pooled for the microarray analysis (with a total of 4 arrays). Fluorescent targets were prepared from 2.5 μg total RNA samples using a miRNA ULSTM Labelling Kit (Kreatech Diagnostics, Amsterdam, Netherlands). Labelled miRNA targets enriched by NanoSep 100K (Pall, Ann Arbor, MI, USA) were hybridized to the Human miRNA OneArray® with Phalanx hybridization buffer using a OneArray® Hybridization Chamber. After 16 h of hybridization at 37 °C, non-specific binding targets were washed away with three different washing steps (wash I at 37 °C for 5 min; wash II at 37 °C for 5 min and 25 °C for 5 min; and wash III which consisted of 20 rinses), and slides were dried by centrifugation and scanned with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensities of each spot were analyzed by GenePix 4.1 software (Molecular Devices). The signal intensity of each spot was process by R program. We filtered out spots with a flag of <0. Spots that passed the criteria were normalized by a 75% media scaling normalization method. Normalized spot intensities were transformed to gene expression $\log_2$ ratios between the control and treatment groups. Spots with a $\log_2$ ratio of ≥1 or of ≤-1 and a p value of <0.05 were used for further analyses.

Because differences in miRNA expressions in subcutaneous adipose tissues between obese men and women were not obvious, we chose to further analyze miRNA in omental adipose tissues.

Prediction of miRNA target genes and biological pathway

Human miRNA target genes were examined using miRBase to predict potential target genes[9] and biological pathways defined by KEGG maps.[10]

Quantitative miRNA by real-time PCR validation methods

To quantify the miRNA expression level by the real-time polymerase chain reaction (PCR) method, complementary (c)DNA was synthesized using an NCode VILO miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Total RNA samples were polyadenlyated and reverse-transcribed using poly A polymerase, ATP, SuperScript III RT, and a specially designed universal reverse-transcription (RT) primer in a single reaction. The cDNA was subjected to real-time PCR using SYBR Green ER qPCR Mix (Invitrogen). Thermocycler conditions were 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 50 s. The forward primer was designed according to the NCode miRNA Database at http://escience.invitrogen.com/ncode. MicroRNA u6 was the endogenous control.

Target genes were analyzed by a quantitative (q)PCR. Total RNA was isolated from adipose tissues using the Trizol reagent (Invitrogen) according to the manufacturer's recommendation. RNA was reverse-transcribed using oligo (dT) 18 primers with a cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). A real-time RT-PCR was carried out in optical 96-well plates on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study included the insulin receptor (INSR; forward (F)-5'TGCCGAGGA CCCTAGGCCTA-3' and reverse (R)-5'GGCACGGCCA CCGTCACATT-3'), insulin receptor substrate 1 (IRS1; F-5'ACGTGCGCAAGGTGGGCTAC-3' and R-5'CGTT TGGGGGCGCTCGACTT3'), phosphoinositide-3-kinase (PI3K; F-5'AGGGAGTCGCCGCTGGAGAG-3' and R-5'CATGTCGTGGCCCCTGGCTG-3'), c-Jun N-terminal kinase (JNK; F-5'GCAGTGATCAATGGCTCTCAGCA-3' and R-5'TACAGCAGCCCAGAGGCCCA-3'), and β-actin (F5'CATGTACGTTGCTATCCAGGC-3' and R-5'CTCCTTAATGTCACGCACGAT-3'). The expression of each gene was assayed in a total volume of 25 μL containing 1× SYBR green master mix reagent (Applied Biosystems), 100 nM of each primer, and 50 ng of cDNA. Amplification was performed according to the thermocycling protocol recommended by the PCR system, with a final dissociation curve (DC) analysis. All samples were analyzed in triplicate, and multiples of change of messenger (m)RNA were calculated as the difference in threshold cycles (ΔCt) between the test gene and GAPDH.

Statistical analysis

Data are expressed as the mean±SD. A t-test was used for the difference between two groups. Pearson's correlation coefficients were used to calculate the linear relationship of miRNA with insulin-related gene expressions and biochemical parameters.

RESULTS

Characteristics of obese subjects

Men had significantly greater height, body weight, AC, HbA1C, TG, waist circumference, and muscle mass than women, whereas women had a significantly higher fat mass than men. There were no differences in the BMI, cholesterol, HDL-C, or hip circumference between the genders (Table 1). No differences in the BMI or lipid profiles were observed between the DM and non-DM groups (BMI, 41.9±1.7 vs. 43.4±2.2 kg/m^2; TG 150.0±21.1 vs. 150.8±64.4 mg/dL; Cholesterol, 201.2±31.2 vs. 195.9±22.4 mg/dL; HDL-C, 46.6±7.9 vs. 45.9±8.5 mg/dL, $p>0.05$). The T2DM group had higher AC levels and HbA1C percentage than the non-DM group (AC, 170.2±64.2 vs. 90.4±8.13 mg/dL; A1C, 8.12±2.09 vs. 5.93±0.46%, $p<0.05$).

Microarray analysis

Differences in miRNA expressions between obese men and women were compared. The top-ranking miRNA in abdominal omental adipose tissues was miRNA-125a-3p ($\log_2$ ratio=2.4). MiR-125a-3p expressed by abdominal omental adipose tissues was much higher in obese men than women. There were no differences in miRNA-125a-3p expressions in subcutaneous adipose tissues between the genders.

MiRNA-125a-3p and other related gene expressions in intra-abdominal omental adipose tissues

We confirmed the expression of miRNA-125a-3p by an RT-PCR. The ΔCt of miRNA-125a-3p was significantly higher in the T2DM group than the non-DM group (10.69±1.23 vs. 12.19±0.89, multiple of change 2.8, $p<0.05$). Obese men had significantly lower miRNA-125a-3p, higher insulin receptor, and lower JNK mRNA expression than women. There were no differences in IRS or PI3K between obese men and women ($p>0.05$) (Table 2).

Correlations of miRNA-125a-3p with insulin signalling-related genes, biochemical parameters and body composition

There was a significant positive correlation between ΔCt of miRNA125a-3p and JNK, whereas significant negative correlations were observed between ΔCt of miRNA125a-3p and the insulin receptor and PI3K in obese men. Only the insulin receptor was significantly associated with miRNA125a-3p in obese women. Plasma AC, cholesterol levels, HbA1C percentage, and the waist/hip ratio were significantly negatively associated with ΔCt of miRNA125a-3p in obese men. The associations with these parameters were not obvious in women. There were significant associations of miRNA125a-3p expression with the waist circumference and fat mass percentage in both obese men and women (Table 3).

DISCUSSION

Several studies demonstrated the regulation of miRNA in adipose tissues and during adipogenesis of preadipocytes

and human adipose-derived stem cells.[5] In this study, using a microarray analysis, we found that miR-125a-3p expression was much higher in abdominal omental adipose tissues in morbidly obese men than women, while no difference was observed in subcutaneous adipose tissues between the sexes. In general, men have preferential abdominal fat accumulation, while women accumulate fat in the gluteal and femoral regions.[4] In this study, although the fat mass was higher in women, we did observe a higher waist circumference in men than women.

Table 1. Characteristics of the obese subjects

	Men(N=11)	Women (N=10)
Age (yr)	33.7±8.6	35.8±9.9
Height (cm)	176±6.7*	164±7.1
BW (kg)	138±12.2*	115±14.8
BMI (kg/m^2)	43.8±2.5	42.4±1.5
AC (mg/dL)	124±30.8*	96.6±14.3
Hemoglobin A1C (%)	6.6±0.5*	5.9±1.9
Triglyceride (mg/dL)	192±67.7*	123±21.6
Cholesterol (mg/dL)	206±33.9	194±12.9
HDL-C (mg/dL)	45.9±6.2	45.6±9.4
Waist circumference (cm)	131±5.3*	119±8.6
Hip circumference (cm)	132±8.4	129±8.1
Waist/hip ratio	1.02±0.03	1.1±0.1
Fat/BW (%)	43.8±5.1*	49.1±3.1
Muscle/BW (%)	33.8±5.6*	28.7±1.7

Data are presented as the mean±SD. *Significantly differs from females (p<0.05).
BW, body weight; BMI, body mass index; AC, fasting glucose; HDL-C, high-density lipoprotein cholesterol.

Table 2. Changes in the threshold cycle (ΔCt) of miRNA125a-3p and insulin signalling-related gene mRNA expressions between male and female obese subjects

ΔCt	Men (N=11)	Women (N=10)	Multiples of change (M/F)
miRNA125a-3p	10.6±0.9*	11.8±0.8	2.3
INSR	12.2±2.8*	9.5±0.4	0.158
IRS1	8.33±1.6	7.8±1.1	0.689
PI3K	10.0±1.2	10.3±0.9	1.2
JNK	6.78±1.1*	8.2±0.2	2.6

Data are presented as the mean±SD. *Significantly differs from females (p<0.05).
INSR, insulin receptor; IRS1, insulin receptor substrate 1; PI3K, phosphoinositide-3-kinase; JNK, c-Jun N-terminal kinase.

Table 3. Correlations of changes in the threshold cycle (ΔCt) of miRNA125a-3pexpression with insulin signalling related genes, biochemical parameters and body composition in obese subjects

	Men(N=11)		Women(N=10)	
	r	p value	r	p value
Insulin signalling-related genes				
INSR	-0.89	0.006*	0.76	0.008*
IRS1	-0.42	0.30	-0.38	0.27
PI3K	-0.81	0.014*	-0.43	0.22
JNK	0.72	0.04*	0.18	0.60
Biochemical parameters				
AC	-0.76	0.04*	-0.03	0.94
Hemoglobin A1C	-0.92	0.02*	-0.05	0.89
Cholesterol	-0.76	0.04*	-0.16	0.65
Triglyceride	-0.56	0.24	-0.11	0.76
HDL-C	-0.46	0.25	0.64	0.04*
Body composition				
BMI	-0.31	0.72	-0.34	0.34
Waist circumference	-0.81	0.02*	-0.73	0.04*
Hip circumference	-0.43	0.33	-0.51	0.87
Waist/hip ratio	-0.76	0.02*	-0.56	0.11
Fat/BW (%)	-0.85	0.02*	-0.72	0.02*
Muscle/BW (%)	0.48	0.21	0.29	0.43

INSR, insulin receptor; IRS1, insulin receptor substrate 1; PI3K, phosphoinositide-3-kinase; JNK, c-Jun N-terminal kinase; AC, fasting glucose; HDL-C, high-density lipoprotein cholesterol; BMI, body mass index; BW, body weight.
*Significantly correlated with miRNA125a-3p expression.

A previous study showed that high level of visceral fat is an independent risk factor for glucose intolerance and insulin resistance,[11] whereas subcutaneous fat might not have a direct impact on metabolic dysfunction.[12] Our finding links miR-125a-3p with abdominal obesity and insulin resistance in men. A previous report revealed that miRNA-125a was overexpressed in liver and adipose tissues in a rat model of T2DM.[7] We also observed that obese subjects with T2DM had higher miR-125a-3p expression in abdominal omental adipose tissues than those with normal blood glucose levels. Because the subject numbers were limited in this study, the sex difference of miR-125a-3p expression in diabetes with severe obesity requires further investigation.

Insulin resistance is a common problem associated with obesity. Insulin signalling initiates with the binding of insulin to its receptor. Insulin induces tyrosine phosphorylation of the insulin receptor and IRS. IRS-1 is a key molecule in the insulin signalling pathway. Failure to activate IRS-1 leads to systemic insulin resistance.[13] Tyrosine-phosphorylated IRS proteins bind the regulatory subunit of PI3K. Activated PI3K recruits and activates downstream molecules.[14] A number of mechanisms might contribute to dysregulation of the insulin signalling pathway, one of which is serine phosphorylation of the insulin receptor or IRS proteins.[15] IRS proteins contain many potential serine or threonine phosphorylation sites that may play regulatory roles during the insulin response.[15] Serine phosphorylation of IRS-1 inhibits the insulin signalling cascade that leads to insulin resistance.[16] JNK is one of the target genes of miR-125a-3p. JNK is a member of the MAPK family and is activated by proinflammatory cytokines, free fatty acids and factors associated with obesity-induced activity.[17] A previous study found that JNKs can interfere with insulin's actions in cultured cells. JNK is a crucial mediator of obesity and insulin resistance.[18] JNK activity was found to be abnormally elevated in obesity. Activation of JNK inhibits the insulin signalling cascade partly by stimulating serine phosphorylation of IRS-1.[15] An absence of JNK1 improved insulin sensitivity and enhanced the insulin receptor signalling capacity.[17] In this study, we found that concomitant with higher miR-125a-3p, JNK was also higher, whereas insulin receptor expression was lower in obese men. These results possibly indicate that miR-125a-3p upregulates JNK, which may inhibit insulin receptor expressions. Our findings also showed a strong positive correlation between miR-125a-3p and JNK, but negative associations between miR-125a-3p and the insulin receptor and PI3K in obese men. These results support the involvement of miR-125a-3p in the insulin signalling pathway and imply that the extent of insulin resistance is more severe in obese men possibly due to the increased JNK activity that is up-regulated by miR-125a-3p.

In this study, associations of miR-125a-3p with insulin signalling-related gene expressions and biochemical markers were prominent in men with morbid obesity; however, the relationship was not obvious in obese women. It is well recognized that women have more body fat than men at the same relative BMI.[18] However, adipose tissue distribution patterns differ between the sexes. The typical male type accumulates in the abdominal cavity instead of the typical peripheral obesity commonly observed in women. In addition, the abdominal type of obesity is associated with adipocyte hypertrophy.[4] Larger adipocytes are more insulin-resistant and lipolytic, and release more inflammatory cytokines and less adiponectin that are more susceptible to metabolic disorders.[19] We did observe that with the same degree of obesity, men had higher blood TG, fasting glucose, and HbA1C than women. This result was consistent with a previous report; those authors also found higher lipid and glucose profiles in obese men and differences in biochemical markers between the sexes persisted even after body fat matching.[4] Since the higher ΔCt of miR-125a-3p indicates lower miR-125a-3p expression in adipose tissues, the results of this study showed that the higher the miR-125a-3p expression was, the higher the plasma glucose and cholesterol levels in men were. Overexpression of miR-125a-3p results in insulin resistance that may consequently lead to abnormal lipid and carbohydrate metabolism. We found that miR-125a-3p expression was associated with the waist circumference and fat mass in both men and women with morbid obesity. This result indicates that a male risk profile may also be seen in women with excess body fat possibly characterized by abdominal obesity.

Noteworthy, our study has limitations. The number of subjects was relatively small. Also, there were no control samples with normal BMI because adipose tissues cannot be obtained from normal subjects. The associations among antropometric and metabolic parameters and miRNA-125a-3p expression between obese and normal weight subjects cannot be known.

In conclusion, this study showed for the first time, that the expression of miR-125a-3p in abdominal omental adipose tissues was higher in obese men than women. Also, the gene expression of JNK was higher while that of the insulin receptor was lower in obese men. MiR-125a-3p was found to be associated with insulin signalling genes and plasma glucose and cholesterol. These associations were not obvious in women with severe obesity. Our results support the involvement of miR-125a-3p in insulin resistance and the insulin signalling pathway and imply that increased miR-125a-3p expression may be a characteristic feature of insulin resistance in obese men. These results are promising, but need to be reconfirmed with larger sample size.

AUTHOR DISCLOSURES

The authors declare that they have no competing interests.

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Original Article

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重度肥胖者腹腔脂肪組織 microRNA-125a-3p 的表現量與胰島素訊息傳遞相關基因表現相關: 台灣人的觀察研究

背景：微形核糖核酸(miRNA)在調節脂肪生合成及胰島素抗性扮演重要角色，但是肥胖者腹腔脂肪組織 miRNA 與胰島素訊息傳遞相關基因表現之間的相關性仍不確知。方法: 本研究利用微陣列的平台來篩檢不同性別重度肥胖者腹腔脂肪組織中 miRNA 的表現，發現在腹腔網膜脂肪表現量最高的是 miRNA-125a-3p，所以我們再用即時聚合酶鏈鎖反應確認並定量所有肥胖者(11 位男性及 10 位女性)腹腔網膜脂肪中 miRNA-125a-3p 及胰島素訊息傳遞相關基因的表現，同時也以線性迴歸法分析 miRNA-125a-3p 與胰島素訊息傳遞相關基因表現及血中生化指標間的相關性。結果: 肥胖男性腹腔網膜脂肪 miRNA-125a-3p 的表現較女性為高，腹腔皮下脂肪則無性別間之差異，有高 miRNA-125a-3p 表現量的肥胖男性，其 c-Jun N-terminal kinase 的基因表現量也會高，而胰島素接受器的表現量則較肥胖女性為低。miRNA-125a-3p 與胰島素接受器及 phosphatidylinositol 3-kinase 的表現成負相關，而空腹血糖和膽固醇值與 miRNA-125a-3p 的表現則成正相關。這些相關性在肥胖男性很顯著，但在肥胖女性則不明顯。結論:本研究結果顯示 miRNA-125a-3p 參與了胰島素訊息傳遞路徑的調節，而腹腔網膜脂肪 miRNA-125a-3p 表現量增加可能是肥胖男性胰島素抗性的一個表徵。

關鍵字：重度肥胖、腹部脂肪組織、miRNA-125a-3p、胰島素訊息路徑、胰島素抗性