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Mechanism of fibroblast growth factor 21 resistance in human fat from patients with insulin resistance

사람지방조직을 중심으로 한 fibroblast growth factor 21 resistance 의 기전에 관한 연구

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Abstract

Mechanism of fibroblast growth factor 21 resistance in human fat from patients with insulin resistance

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Ectopic fat accumulation is a typical feature in metabolic deterioration conditions such as diabetes, metabolic syndrome, and cardiovascular disease. Fibroblast growth factor 21 (FGF21) is a novel metabolic regulatory protein produced primarily by the liver and acts mainly through the adipose tissue; it regulates glucose and lipid metabolism. However, plasma FGF21 concentrations are

paradoxically increased in type 2 diabetes mellitus (T2DM) and obesity, suggesting resistance to the action of this ligand.

This study aimed to investigate the relationship between FGF21 concentrations and various types of ectopic fat accumulation in patients with insulin resistance. Furthermore, this study aimed to elucidate the mechanism underlying FGF21 resistance in T2DM patients by investigating FGF21 receptor expression and post-receptor signaling in different fat deposits.

Three independent cohorts were investigated. Cohort 1 consisted of 190 patients referred from a health promotion center. Based on a review of their medical history, demographics, clinical profiles, and concomitant medication, the patients were identified and analyzed for normal glucose tolerance (NGT), prediabetes, and T2DM. Sixty-four-slice multi-detector computed tomography was used to quantify fat amount from various sites (subcutaneous, visceral, epicardial, intrahepatic, and intramuscular fat) and plasma FGF21 concentrations were measured. Visceral and subcutaneous adipose tissues were obtained from patients undergoing coronary artery bypass surgery (Cohort 2) and patients undergoing general

abdominal surgery (Cohort 3). FGF21 receptor expression and post-receptor signaling in different fat deposits of both non-diabetic and T2DM patients were analyzed.

Plasma FGF21 concentrations were significantly associated with body mass index, triglyceride concentrations, homeostatic model assessment of insulin resistance, and the Matsuda index. Plasma FGF21 concentrations were significantly higher in T2DM patients than in the prediabetic and NGT groups. Similarly, the amount of ectopic fat (visceral, epicardial, intrahepatic, and intramuscular fat) in patients with T2DM was significantly higher than that in the NGT group, and plasma FGF21 concentrations were strongly correlated with ectopic fat accumulation in T2DM patients. Protein expression of the FGF receptor dimer (FGFR1 and β-klotho) and post-receptor signaling pathway-related protein (p-P38) was lower in visceral fat than in subcutaneous fat in patients of T2DM.

In conclusion, plasma FGF21 concentrations are higher in patients with T2DM. Plasma FGF21 is positively correlated with ectopic fat accumulation in this disease. FGF21 receptor and post-receptor signaling protein expression is attenuated in visceral fat. Thus,

human FGF21 resistance in T2DM could result from downregulation of the FGF21 receptor dimer and post-receptor signaling in ectopic fat accumulation. These findings will help understand the mechanism underlying FGF21 resistance and provide clinical insights that may aid the development of FGF21-based therapeutics in diabetes.

Keywords: fibroblast growth factor 21, ectopic fat, type 2 diabetes, FGF21 resistance

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Introduction

The fibroblast growth factors (FGF) are a family of cell signaling proteins that are involved in a wide variety of processes, most notably as crucial elements for normal development. These growth factors generally act as systemic or locally circulating molecules of extracellular origin that activate cell surface receptors. While the majority of the 22 known members of FGF family have been primarily associated with mitosis, development, transformation, angiogenesis, and survival (McKeehan, Wang et al. 1998), some FGFs play important roles in defining and regulating functions of some endocrine-relevant tissues and organs, as well as modulating various metabolic processes. FGF15/19, FGF21 and FGF23 are hormone-like FGFs that have systemic effects (Fukumoto 2008).

FGF21 is a novel member of FGF family. It is preferentially expressed in liver and acts mainly through white adipose tissue (Badman, Pissios et al. 2007, Inagaki, Dutchak et al. 2007). Genomic studies allowed the identification of the cDNA encoding a 209 amino acid nucleotide sequence of the human FGF21

(Nishimura, Nakatake et al. 2000). The human DNA sequence of FGF21 is highly identical (75%) to mouse FGF21. The molecular mechanism of FGF21 signaling is complex and involves several FGF receptors (FGFRs) as well as an obligate co-receptor β-klotho. Tissue specificity of FGF signaling is conferred by the co-expression of a given FGFR and β-klotho. FGFR1 has the highest affinity for FGF21. The C terminus of FGF21 interacts with β-klotho and the N terminus of FGF21 interacts with the extracellular ligand binding domains of the FGFR1 receptor (Yie, Wang et al. 2012). In addition, photobleaching experiments have found that β-klotho and FGFR1 aggregate to form heterodimeric complexes on the cell membrane at a 1:1 ratio.

Several studies in vitro and in vivo showed that FGF21 is an important modulator of insulin and glucose metabolism. In cultures of human adipocytes and in 3T3-L1 cells, FGF21 administration has a dose-dependent effect on stimulating glucose incorporation (Kharitonenkov, Shiyanova et al. 2005). In vivo studies using the obese or diabetic rodents have detected that FGF21 treatment increases expression of glucose transporter 1 specifically on adipose tissue, islet insulin content and glucose induced insulin

secretion (Kharitonenkov, Shiyanova et al. 2005, Wente, Efanov et al. 2006). In addition to the reduction of glucose levels, FGF21 administration in obese mice and diabetic monkeys led to significant improvements in triglyceride (TG) level and lipoprotein profiles (Kharitonenkov, Shiyanova et al. 2005, Kharitonenkov, Wroblewski et al. 2007). FGF21 has also been related with the fasting and energy balance. For example, the therapeutic administration of FGF21 in obese mice increased energetic waste, body temperature, fat utilization, and reduced hepatic steatosis, associated with body weight reduction (Coskun, Bina et al. 2008, Xu, Lloyd et al. 2009).

These beneficial effects of FGF21 suggest that FGF21 or an agonist could be a potential therapeutic agent for diabetes. In fact, drug discovery efforts have yielded a FGF21 analog (Zhao, Dunbar et al. 2012), but its effect on glycemic control was not as robust as anticipated based on prior experiments using diabetic rodents. Another FGF21 analog was shown to decrease body weight and improve lipid profiles in subjects with type 2 diabetes mellitus (T2DM) without significant effects on glycemic control (Talukdar, Zhou et al. 2016).

However, several human studies have revealed that plasma FGF21 levels are paradoxically increased in T2DM (Chen, Li et al. 2008, Chavez, Molina-Carrion et al. 2009) and insulin-resistant states such as nonalcoholic fatty liver disease (Dushay, Chui et al. 2010). Furthermore, FGF21 concentrations are positively correlated with body mass index (BMI) or TG levels in obese non-diabetic individuals (Zhang, Yeung et al. 2008). Serum FGF21 levels were also found to be significantly higher in patients with end-stage renal disease and coronary heart disease (Han, Choi et al. 2010, Lin, Wu et al. 2010). It has been suggested that this paradoxical increase in plasma FGF21 levels could be a result of "FGF21 resistance" (Kharitonenkov and Larsen 2011). In a previous study, FGF21 levels are also increased in mice with diet-induced obesity (DIO). In response to FGF21, obese mice demonstrate marked deficits in the ability of the peptide to initiate signals through the ras-raf-MAPK cascade, as demonstrated by the remarkably low levels of ERK1/2 phosphorylation in liver and white adipose tissue. (Fisher, Chui et al. 2010). The results of this animal study and the paradoxical increase in plasma FGF21 in patients with T2DM support the hypothesis that FGF21 resistance might play a role in obesity and T2DM.

In contrast to orthotopic subcutaneous fat, ectopic fat is defined as the deposition of TG in ectopic sites or within cells of non-adipose tissue. Fat tissues in ectopic sites include visceral and epicardial fat and fat depositions in non-adipose tissue cells include intrahepatic and intramuscular fat (Gastaldelli and Basta 2010). It is evident that ectopic fat is strongly associated with diabetes, metabolic syndrome, and cardiovascular disease (Perseghin, Scifo et al. 1999, Wajchenberg 2000, Lautamaki, Borra et al. 2006, Kim, Yu et al. 2011). These observations suggest that ectopic fat plays a major pathogenic role in T2DM. However, little is known regarding the relationship between FGF21 and ectopic fat or the role of ectopic fat in FGF21 metabolism.

The aim of the present study was to investigate the relationship between FGF21 concentrations and various types of ectopic fat accumulation. Furthermore, this study aimed to identify the mechanism underlying paradoxical FGF21 elevation in T2DM subjects by investigation of FGF21 receptor expression and post-receptor signaling in different fat deposits of both non-diabetes and T2DM subjects.

Research design and methods

Subjects

Three independent cohorts were investigated. Cohort 1 was enrolled to investigate the association between plasma FGF21 levels and ectopic fat phenotypes. Cohort 2 and 3 were enrolled to investigate fat tissue FGF21 signaling-related gene expression profiles. This study was approved by the local ethics committee (SNUBH IRB#B-1203/147-006, #A111218-CP02) and all subjects provided written informed consent.

Cohorts are described as follows.

Cohort 1: Subjects referred to Seoul National University Bundang Hospital from a health promotion center and patients who received regular follow-up for prediabetes and drug-naive T2DM were screened. Among them, subjects over 30 years of age who underwent 64-slice multi-detector computed tomography (MDCT) for the assessment of coronary artery disease (CAD) were enrolled. We excluded patients with malignant disease, chronic wasting diseases such as tuberculosis or malabsorption syndrome, advanced liver disease, and advanced renal disease. We excluded patients

with cocaine consumption, chronic steroid use, or anabolic drug use. Finally, 190 unrelated subjects were enrolled in the study. We identified risk factors based on medical history, demographics, baseline clinical profiles, and concomitant medications. We analyzed plasma FGF21 levels and MDCT data for this cohort.

Cohort 2: Subjects who underwent coronary artery bypass graft surgery at Seoul National University Bundang Hospital were enrolled. Plasma FGF21 levels were analyzed in 40 subjects. Visceral (intrathoracic preperitoneal) adipose tissue (VAT) and subcutaneous adipose tissue (SAT) samples were obtained from eight non-diabetic control and eight diabetic patients.

Cohort 3: Subjects who underwent general abdominal surgery at Seoul National University Bundang Hospital were enrolled. For an additional non-diabetic control group, we obtained VAT and SAT from the eight non-diabetic subjects.

Measurement of anthropometric and biochemical parameters

Body weight, height, waist circumference, and blood pressure were measured when subjects were enrolled. BMI was calculated as the

weight in kilograms divided by the square of the height in meters (kg/m^2). The concentrations of total cholesterol, TG, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), aspartate transaminase (AST), alanine aminotransferase (ALT), fasting plasma glucose (FPG) after at least 12-h fast were measured using the Toshiba 200FR Neo chemistry autoanalyser (Toshiba Medical Systems Co., Ltd,Tokyo, Japan). Glycated hemoglobin (HbA1c) was measured by high performance liquid chromatography using the Bio-Rad Variant II Hemoglobin Testing System (Bio-Rad Laboratories, Munich,Germany). Plasma insulin concentrations were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) with anti-rat insulin antibody (Linco Research, St Charles, MO, USA). Postprandial 30-min and 2-h glucose (PP2) levels were sampled during a 75-g oral glucose tolerance test (OGTT) in cohort 1. We defined NGT, prediabetes, and T2DM according to the definition of American Diabetes Association. We calculated insulin resistance with the homeostasis model using the following validated formula: HOMA-IR = (fasting glucose (mg/dL) × fasting insulin (μIU/mL)) / (22.5 × 18) (Bonora, Targher et al. 2000). In subjects with OGTT, Matsuda index (10000 / square root of [fasting glucose

× fasting insulin] × [mean glucose × mean insulin during OGTT]) was used as the insulin sensitivity index (Matsuda and DeFronzo 1999). Plasma FGF21 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Biovendor, Germany) with samples frozen at −80 ° C until the assay was performed.

Computed tomography protocol

A 64-slice MDCT was used to detect CAD during a routine health examination. Subjects with a heart rate > 70 beats per min received 10~30 mg of intravenous esmolol (Jeil Pharm, Seoul, Korea) before MDCT imaging. CT angiography was performed with a 64-slice MDCT scanner (Brilliance 64, Philips Medical Systems, Best, Netherlands). A standard scanning protocol described previously was employed (Choi, Choi et al. 2008). A 10-mm CT slice image of the abdomen (L4-L5) and a mid-thigh level were added to this protocol to assess other ectopic fat accumulation.

Fat assessment

Abdominal subcutaneous and visceral fat areas were assessed at the L4-L5 intervertebral disc space within a threshold of −250 to

−50 Hounsfield units (HU) with a dedicated computer 3D workstation (Rapidia 2.8, Infinitt, Seoul, Korea) (Kim, Nam et al. 2003).

Epicardial fat volume was measured by a single observer with the same software. Epicardial fat volume was segmented by isolating the epicardial fat and heart from the thorax using specific anatomical landmarks. The superior extent of the volume was determined by the point initiating the division of the main pulmonary artery. Inferiorly, the analysis volume was segmented from the liver and abdominal cavity by manually tracing the pericardium in the axial view every 5 mm from the top to bottom with software that automatically interpolated between the user−defined traces (Wheeler, Shi et al. 2005). After segmentation of the heart and epicardial adipose tissue from the remainder of the thorax, a threshold of −190 to −30 HU was applied to isolate adipose tissue−containing voxels. The adipose tissue voxels were then summed to provide epicardial fat volume value in milliliters. The examinations were placed in random order with the observer blinded to other participant information.

Non−contrast CT was used to measure intrahepatic fat. Images were reviewed by a single observer blinded to other data. CT

attenuation of three distinct circular areas was measured in addition to the spleen to generate mean values. Care was taken to avoid the inclusion of visually distinct vasculature and biliary structures in the regions of interest. Liver-spleen HU differences were calculated as the mean hepatic HU - mean splenic HU (Shores, Link et al. 2011). Lower liver-spleen HU differences were considered to indicate higher intrahepatic fat accumulation.

Skeletal muscle attenuation was determined by measuring the mean value of all pixels within the range of 0 to +100 HU. The mid-thigh skeletal muscle area was compartmentalized into a normal density muscle area (+31 to + 100 HU) and a low-density muscle area (0 to +30 HU) (Kim, Nam et al. 2003). The low-density muscle area indicated an intramuscular fat area.

Fat tissue samples

During surgery, biopsies of adipose tissues were obtained after an overnight fast, washed in 9 g/L NaCl solution, sectioned into pieces, immediately frozen in a deep freezer, and stored −40 ° C. In cohort 2, visceral fat sample was obtained from the intrathoracic preperitoneal fat which is located in the fat compartment under xiphoid process from the anterior surface of the left lobe of the

liver. Subcutaneous fat sample was obtained from the incision field. In cohort 3, visceral fat sample was obtained during laparoscopic surgery. Subcutaneous fat sample was obtained from the incision field. The surgeon aimed to obtain the samples from similar anatomical locations in all the subjects.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from frozen tissue samples using TRIzol (Ambion, CA, USA). For quantitative real-time PCR analysis, 3 μg of total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, CA, USA). SYBR Green reactions using the SYBR Green PCR Master mix (Enzynomics, Korea) were assembled along with 10 pM primers according to the manufacturer's instructions and were reactions were performed using the Applied Biosystems ViiA7 system (Thermo Scientific, CA, USA). Relative mRNA levels were calculated using the comparative CT method and normalized to *cyclophilin* mRNA. mRNA expression levels are displayed relative to those in visceral fat as a control. The sequences of all primers used are listed in Table 1.

Table 1. Primer sequences for real time qPCR

Primers	Forward	Reverse
FGF21	AGATGCGGTCGCTTCTTTCA	TGCGCCCCATCTGAATTTCT
FGFR1C	ACAAGATGCTCTCCCCTCCT	GGGCATACGGTTTGGTTTGG
KLB	CCAAACCGGTCGGAAAACAC	CACATCTGGCGTGGACTCTT
PPARγ	TCTCAAACGAGAGTCAGCCTT	CACGGAGCTGATCCCAAAGT
AdipoQ	GCAGTCTGTGGTTCTGATTC	GCCCTTGAGTCGTGGTTTCC
aP2	GCCTACACCCAGAGCTACCG	GCCATGGTACTTGGCCTTG
FASN	TACTGGGCCAGGAATTTGAC	GTGGAAGTGACGCCTTTCAT
Plin1	CAGACCACCATGCACCTG	GCTGTTCTTGTCCACCGACT
ATGL	GTCAAGAGCAAGGCCAAGAA	AGCTGCTCCACCTTCTTCTG
HSL	AAGGCCTACAGGTGCAGTTC	CCAGATTGTTGCAGCGGTTC
LPL	ACCTGCCTTACATGGCTTGTT	CACGCCCTTCTCATAGGCAT
CEBPα	GTATGTGACCGCCTGCTTACT	GCAGGTTCCAAATGCCAGG
CEBPβ	TGCTAGGCACATAGCCTCCT	GCTGGGCTATGGGTGTCTTT
FAS	GCCTACACCCAGAGCTACCG	GCCATGGTACTTGGCCTTG
Cyclophilin	TCTGCACTGCCAAGACTGAG	TCGAGTTGTCCACAGTCAGC

Western blotting

In brief, tissues were homogenized in tissue lysis buffer (Cell Signaling, MA, USA) supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride). Protein concentrations were determined with the Bradford protein assay (Amresco, OH, USA). Then, 20 μg of protein was analyzed by SDS-PAGE on a 10% Tris/HCl gel and transferred onto nitrocellulose membranes (Whatman, USA). Blotting with anti-FGFR1 (Abcam, UK), anti-β-klotho (R&D, MN, USA), anti-p-ERK (cell signaling, MA, USA), anti-p-P38 (Santa Cruz, Germany), and anti-β-actin (Sigma-Aldrich, MO, USA) antibodies was performed and the blots were developed with ECL prime (Amersham, USA). Results are expressed as fold-change compared to data from visceral fat.

Statistical analysis

All values are expressed as means ± SD or medians (interquartile range). For the analysis of variance tests, clinical parameters and regional fat distribution that were not normally distributed were log-transformed to reduce skewedness; we presented the results by taking the anti-logarithm for simple interpretation. Differences in parameters among groups were analyzed by one-way ANOVA or

t-tests. When significantly different values were observed, a Bonferroni post hoc analysis was applied to determine the significance of the relationship between the means. Associations between parameters were identified using Pearson correlations. A p-value less than 0.05 was considered significant. All analyses were performed using SPSS 18.0 for Windows.

Results

Study population and clinical characteristics

According to glucose tolerance, the subjects in cohort 1 were divided three groups as follows: NGT, prediabetes, T2DM. The clinical characteristics for the groups are shown in Table 2. Eighty-nine (47%) subjects were classified as having T2DM, 53 (28%) subjects were classified as having prediabetes, and 48 (25%) subjects were in the NGT group. Age and blood pressure were similar but sex was significantly different among the three groups. BMI was significantly higher in the T2DM and prediabetes groups than in the NGT group. Based on lipid profiles, TG levels were significantly higher and HDL-C levels were significantly lower in the T2DM groups than in the NGT group. All glucose metabolic parameters including FPG, PP2, HbA1c, fasting insulin, fasting c-peptide, and HOMA-IR levels were significantly higher for T2DM and prediabetes patients compared to those in the NGT groups. The Matsuda index was significantly lower in the T2DM and prediabetes groups than in the NGT group.

Table 2. Basal characteristics of subjects (cohort 1)

	NGT (n=48)	Prediabetes (n=53)	T2DM (n=89)	*p-value*
Age (years)	57.8 ± 7.5	57.6 ± 10.4	56.4 ± 9.3	0.606
Male: Female	19 : 29	38 : 15	62 : 27	0.001
WC (cm)	88.5 ± 8.8	89.7 ± 6.0	89.7 ± 8.0	0.813
BMI (kg/m^2)	24.1 ± 2.8	25.4 ± 2.9*	25.5 ± 2.6†	0.016
SBP (mmHg)	124.5 ± 11.6	128.1 ± 15.6	127.7 ± 13.0	0.353
DBP (mmHg)	76.8 ± 8.2	77.3 ± 11.0	79.0 ± 10.4	0.418
T. chol. (mg/dL)	214.6 ± 30.5	212.8 ± 41.1	211.5 ± 47.7	0.718
TG (mg/dL)	137.3 ± 78.3	165.7 ± 104.1	233.6 ± 280.9†	0.028
HDL-C (mg/dL)	55.4 ± 13.2	50.1 ± 11.4	48.9 ± 9.2†	0.023
LDL-C (mg/dL)	118.8 ± 26.5	122.2 ± 35.6	113.4 ± 32.0	0.312
AST (IU/L)	23.8 ± 8.2	25.5 ± 11.8	24.9 ± 11.0	0.712
ALT (IU/L)	27.0 ± 22.1	31.0 ± 19.2	32.7 ± 20.9	0.079
FPG (mg/dL)	92.1 ± 5.4	110.5 ± 8.5*	146.9 ± 44.2†‡	< 0.001
PP2 (mg/dL)	134.5 ± 31.6	145.5 ± 29.6	277.0 ± 87.8†‡	< 0.001
HbA1c (%)	5.7 ± 0.3	6.0 ± 0.4*	7.3 ± 1.4†‡	< 0.001
Fasting insulin (μIU/mL)	8.5 ± 3.1	11.9 ± 5.1*	11.2 ± 4.7†	0.003
Fasting c-peptide (ng/mL)	1.4 ± 0.5	2.2 ± 0.9*	2.2 ± 0.9†	< 0.001
HOMA-IR	1.9 ± 0.7	3.3 ± 1.4*	4.1 ± 2.1†	< 0.001
Matsuda index	5.8 ± 1.9	4.3 ± 1.9*	4.1 ± 1.9†	0.002
FGF21 (pg/mL)	102.2 ± 90.6	114.7 ± 107.5	161.2 ± 152.2†	0.041

Data are presented as mean ± SD; One-way ANOVA was used after testing for normality of distribution; WC, waist circumference; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T.chol., total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate transaminase; ALT, alanine aminotransferase; FPG, fasting plasma glucose; PP2, postprandial 2-h glucose; FGF21, fibroblast growth factor 21; * NGT vs. Prediabetes, † NGT vs. Type 2 diabetes, ‡ Prediabetes vs. Type 2 diabetes

Relationship between FGF21 and the clinical parameters

Cohort 1: Plasma FGF21 levels were significantly associated with BMI, TG, HOMA-IR, and Matsuda index ($r = 0.212$, $p = 0.008$; $r = 0.292$, $p = 0.001$; $r = 0.246$, $p = 0.004$; -0.240, $p = 0.015$, respectively; **Table 3)**. The relationships between plasma FGF21 levels and serum glycemic variables such as FPG, PP2, and HbA1c were not significant. However, plasma FGF21 levels were significantly higher in T2DM patients than in the prediabetes and NGT groups (161.2 ± 152.2 vs. 114.7 ± 107.5 and 102.2 ± 90.6 pg/mL, respectively; $p = 0.041$; **Table 2** and **Fig. 1A)**. Plasma FGF21 levels were not significantly different between males and females (129.8 ± 123.1 vs. 137.4 ± 137.6 pg/mL; $p = 0.862$).

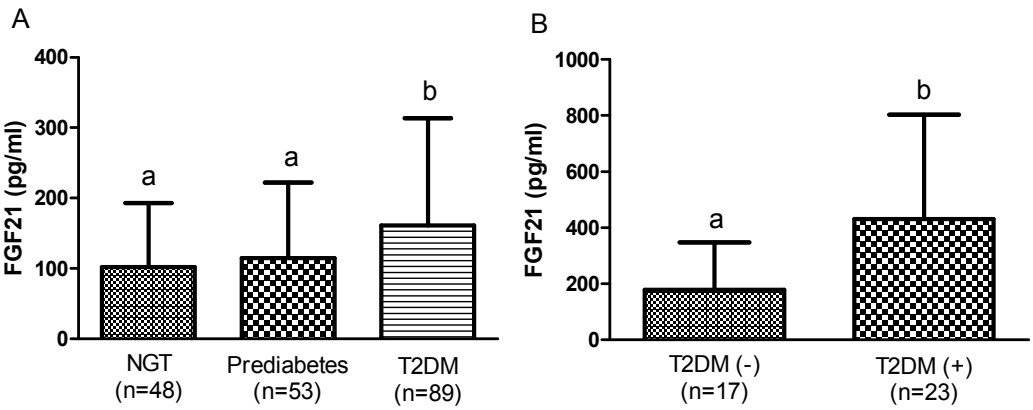
Cohort 2: The clinical characteristics are of this cohort are shown in **Table 4.** Similar to the results of cohort 1, plasma FGF21 levels were significantly higher in the T2DM group than in the control group (431.6 ± 371.1 vs. 178.4 ± 169.4 pg/mL; $p = 0.009$; **Fig. 1B)**.

Table 3. Correlation between FGF21 and clinical parameters

FGF21	r	*p-value*
Age	−.108	.174
BMI	.212	.008
SBP	.123	.125
DBP	.095	.239
T.chol	.137	.089
TG	.292	.001
HDL-C	−.091	.322
LDL-C	.182	.049
AST	.047	.564
ALT	.096	.237
FPG	.145	.069
PP2	.174	.066
HbA1c	.119	.147
HOMA-IR	.246	.004
Matsuda index	−.240	.016

Data were log-transformed before analysis; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T.chol., total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate transaminase; ALT, alanine aminotransferase; FPG, fasting plasma glucose; PP2, postprandial 2-h glucose

Figure 1. Plasma FGF21 levels according to glucose metabolism status.



A: Subjects who referred from a health promotion center (Cohort 1)

B: Subjects who underwent coronary artery bypass graft surgery (Cohort 2)

NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus.

[a, b] Significant differences ($P < 0.05$)

Table 4. Basal characteristics of subjects (cohort 2)

	T2DM (-) (n=17)	T2DM (+) (n=23)	*p-value*
Age (years)	65.4 ± 12.4	68.1 ± 8.6	0.408
Male: Female	15 : 2	16 : 7	0.256
BMI (kg/m^2)	23.6 ± 2.2	24.5 ± 4.0	0.421
SBP (mmHg)	127.7 ± 17.9	123.2 ± 17.7	0.436
DBP (mmHg)	72.0 ± 11.1	69.7 ± 9.9	0.502
T. chol. (mg/dL)	179.5 ± 43.5	164.7 ± 53.5	0.356
TG (mg/dL)	142.7 ± 89.4	144.4 ± 130.0	0.966
HDL-C (mg/dL)	51.5 ± 30.4	43.5 ± 11.5	0.301
LDL-C (mg/dL)	106.4 ± 46.8	92.8 ± 33.2	0.343
AST (IU/L)	35.8 ± 16.5	39.1 ± 26.3	0.886
ALT (IU/L)	25.4 ± 14.2	32.1 ± 20.6	0.246
FPG (mg/dL)	116.6 ± 42.5	185.8 ± 72.3	0.002
HbA1c (%)	5.9 ± 0.7	7.9 ± 1.3	< 0.001
FGF21 (pg/mL)	178.4 ± 169.4	431.6 ± 371.1	0.009

Data are presented as mean ± SD; Student's t-test was used after testing for normality of distribution; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T.chol., total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate transaminase; ALT, alanine aminotransferase; FPG, fasting plasma glucose; ; FGF21, fibroblast growth factor 21

Ectopic fat accumulation

The ectopic fat phenotypes (visceral, epicardial, intrahepatic, and intramuscular fat) of cohort 1 are presented in Table 5. Abdominal visceral fat area was significantly greater in T2DM and prediabetes groups than in subjects with NGT. Epicardial fat volume was significantly greater in subjects with T2DM than in subjects with NGT. Liver-spleen HU differences (intrahepatic fat) were marginally lower in the T2DM group than in NGT subjects, representing higher fat accumulation. Mid-thigh low-density muscle area (intramuscular fat) was also significantly greater in T2DM patients than in subjects with NGT. However, abdominal subcutaneous fat was not significantly different between the groups. Further, each ectopic fat phenotype was strongly associated with each other (Table 6).

Table 5. Ectopic fat accumulation according to glucose metabolism

	NGT (n=48)	Prediabetes (n=53)	T2DM (n=89)	*p-value*
Abdominal subcutaneous fat area (cm^2)	178.9 ± 70.5	171.0 ± 63.3	164.6 ± 67.5	0.390
Abdominal visceral fat area (cm^2)	119.1 ± 53.6	140.0 ± 44.4*	148.1 ± 49.6†	0.001
Epicardial fat volume (cm^3)	83.5 ± 36.0	89.4 ± 35.1	98.6 ± 40.3†	0.042
Liver-Spleen HU difference (HU)	7.2 ± 9.1	4.7 ± 10.5	2.0 ± 11.3	0.059
Mid-thigh low density muscle area (cm^2)	34.2 ± 10.7	41.1 ± 12.4*	41.8 ± 15.3†	0.003

Data are presented as mean ± SD. One-way ANOVA was used after testing for normality of distribution.
* NGT vs. Prediabetes, † NGT vs. Type 2 diabetes, ‡ Prediabetes vs. Type 2 diabetes

Table 6. Correlation among various types of ectopic fat and FGF21 levels

(A) All groups

	Subcutaneous fat	Visceral fat	Epicardial fat	Intrahepatic fat	Intramuscular fat
Subcutaneous fat	1				
Visceral fat	0.206**	1			
Epicardial fat	0.156*	0.612***	1		
Intrahepatic fat	0.226**	0.321***	0.246**	1	
Intramuscular fat	0.399***	0.319***	0.309***	0.112	1
Plasma FGF21	0.029	0.150	0.189*	0.117	0.104

Data were log-transformed before analysis.
*P < 0·05, **P < 0·01, and *** <0.001 by Pearson's correlation analysis.

(B) Normal glucose tolerance

	Subcutaneous fat	Visceral fat	Epicardial fat	Intrahepatic fat	Intramuscular fat
Subcutaneous fat	1				
Visceral fat	0.056	1			
Epicardial fat	-0.129	0.690***	1		
Intrahepatic fat	0.170	0.330*	0.121	1	
Intramuscular fat	0.523***	0.274	0.027	0.106	1
Plasma FGF21	-0.183	0.035	0.130	-0.104	-0.072

Data were log-transformed before analysis.
*P < 0·05, **P < 0·01, and *** <0.001 by Pearson's correlation analysis.

(C) Prediabetes

	Subcutaneous fat	Visceral fat	Epicardial fat	Intrahepatic fat	Intramuscular fat
Subcutaneous fat	1				
Visceral fat	0.225	1			
Epicardial fat	0.147	0.587***	1		
Intrahepatic fat	0.338*	0.198	0.057	1	
Intramuscular fat	0.369**	0.236	0.278	-0.044	1
Plasma FGF21	0.107	0.071	-0.039	0.040	0.017

Data were log-transformed before analysis.
*P < 0·05, **P < 0·01, and *** <0.001 by Pearson's correlation analysis.

(D) Type 2 diabetes mellitus

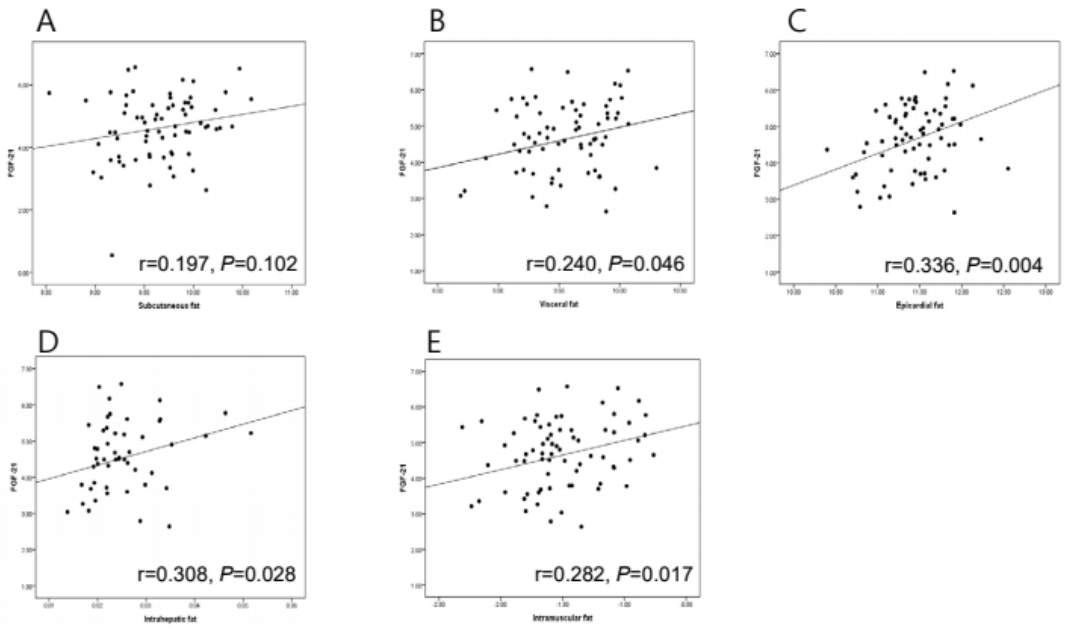
	Subcutaneous fat	Visceral fat	Epicardial fat	Intrahepatic fat	Intramuscular fat
Subcutaneous fat	1				
Visceral fat	0.365**	1			
Epicardial fat	0.367**	0.535***	1		
Intrahepatic fat	0.222	0.311*	0.352**	1	
Intramuscular fat	0.381***	0.367***	0.484***	0.315	1
Plasma FGF21	0.197	0.240*	0.336**	0.308*	0.282*

Data were log-transformed before analysis.
*P < 0·05, **P < 0·01, and *** <0.001 by Pearson's correlation analysis.

Correlation between plasma FGF21 and ectopic fat accumulation

Among regional fat amounts, only epicardial fat was significantly associated with plasma FGF21 levels (r = 0.189, p = 0.019; Table 6A). Based on subgroup analysis of patients with T2DM, all ectopic fat amounts were highly correlated with plasma FGF-21 levels including visceral (r = 0.240, p = 0.046), epicardial (r = 0.336, p = 0.004), intrahepatic (r = 0.308, p = 0.028), and intramuscular (r = 0.282, p = 0.017) fat (Table 6D and Fig. 2). However, subcutaneous fat and plasma FGF21 levels were not significantly associated (r = 0.197, p = 0.102). There were no significant correlations between plasma FGF21 levels and the indices of ectopic fat based on subgroup analysis with NGT and prediabetic subjects (Table 6B and 6C).

Figure 2. Correlation between ectopic fat and FGF21 levels in type 2 diabetes mellitus patients.

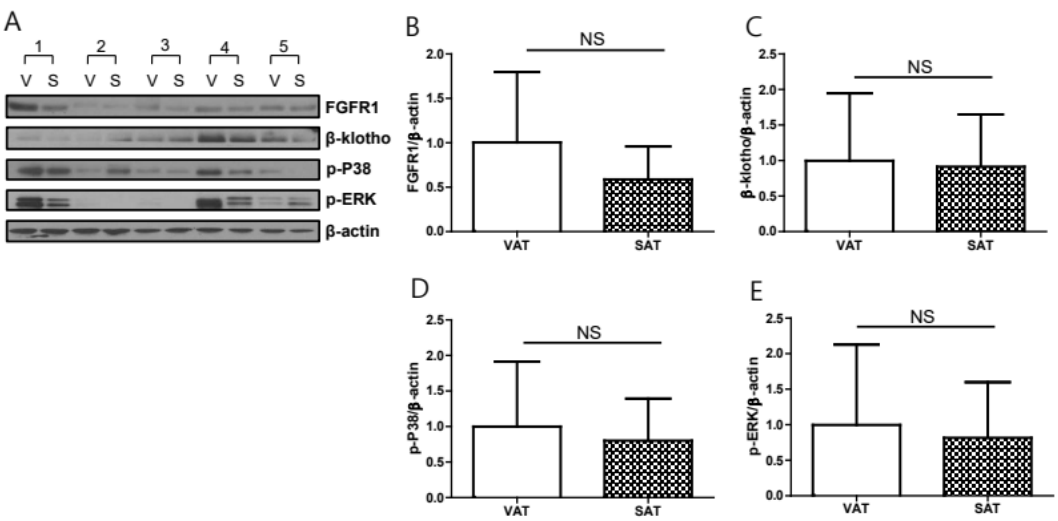


A: Subcutaneous fat, B: Visceral fat, C: Epicardial fat, D: Intrahepatic fat, E: Intramuscular fat.

Alterations in FGF receptor and signaling components in adipose tissue

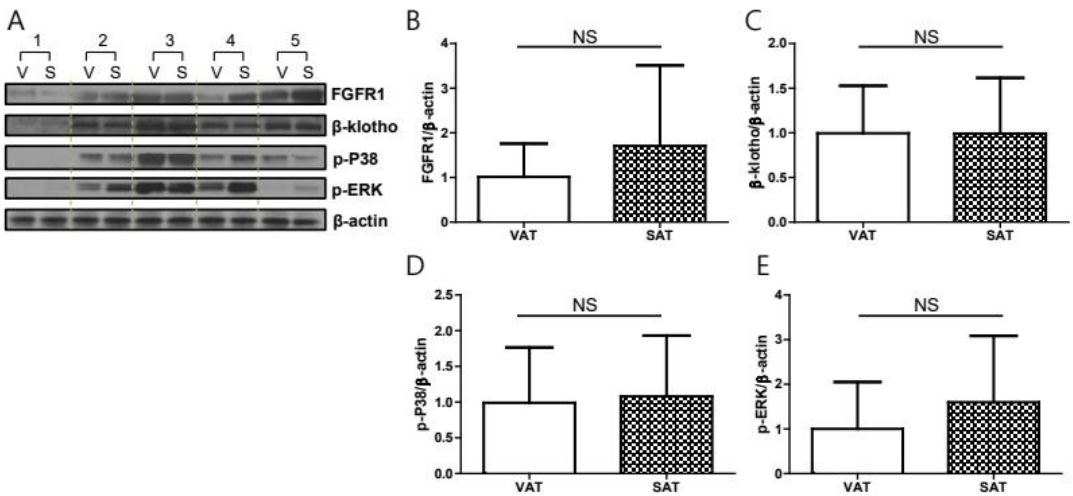
We next investigated protein and RNA levels of FGF21 receptors and post-receptor signaling components in human VAT and SAT. In Cohort 3 (control), protein expression of the FGF21 receptor dimer (FGFR1, β-klotho) and post-receptor signaling pathway-related proteins (p-P38, p-ERK) was not significantly different between the VAT and SAT **(Fig. 3)**. Similar results were found for the adipose tissues of the non-diabetic control group in Cohort 2 **(Fig. 4)**. However, the protein expression of FGFR1, β-klotho, and p-P38 was significantly lower in the VAT than in the SAT of T2DM patients in Cohort 2 **(Fig. 5)**. The expression of p-ERK was variable and not statistically different in each T2DM subject. The gene expression of FGF21 receptor dimer and PPARγ signaling components was not reached to statistical significance between VAT and SAT **(Fig. 6)**. However, it showed similar pattern of changes with respect to the protein expression of FGF21 receptors and post-receptor signaling in T2DM patients.

Figure 3. Protein expression of the FGFR dimer and signaling components in visceral and subcutaneous fat tissue of the non-diabetic control group of subjects who underwent general abdominal surgery (Cohort 3)



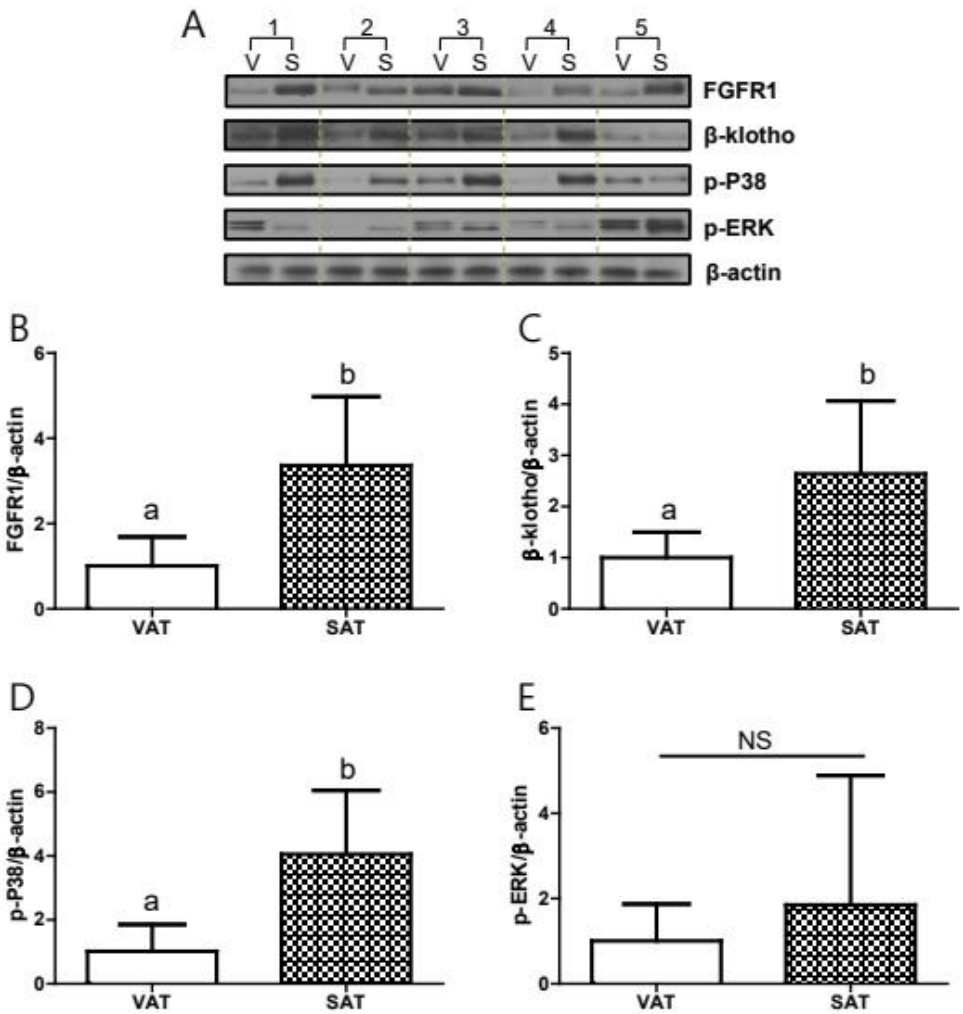
A: Western blot analysis, B: FGFR1, C: β-klotho, D: p-P38, E: p-ERK. V, VAT: visceral adipose tissue, S, SAT: subcutaneous adipose tissue. n=5/group. NS: nonsignificant.

Figure 4. Protein expression of the FGFR dimer and signaling components in visceral and subcutaneous fat tissue of the non-diabetic control group of subjects who underwent coronary artery bypass graft surgery (Cohort 2)



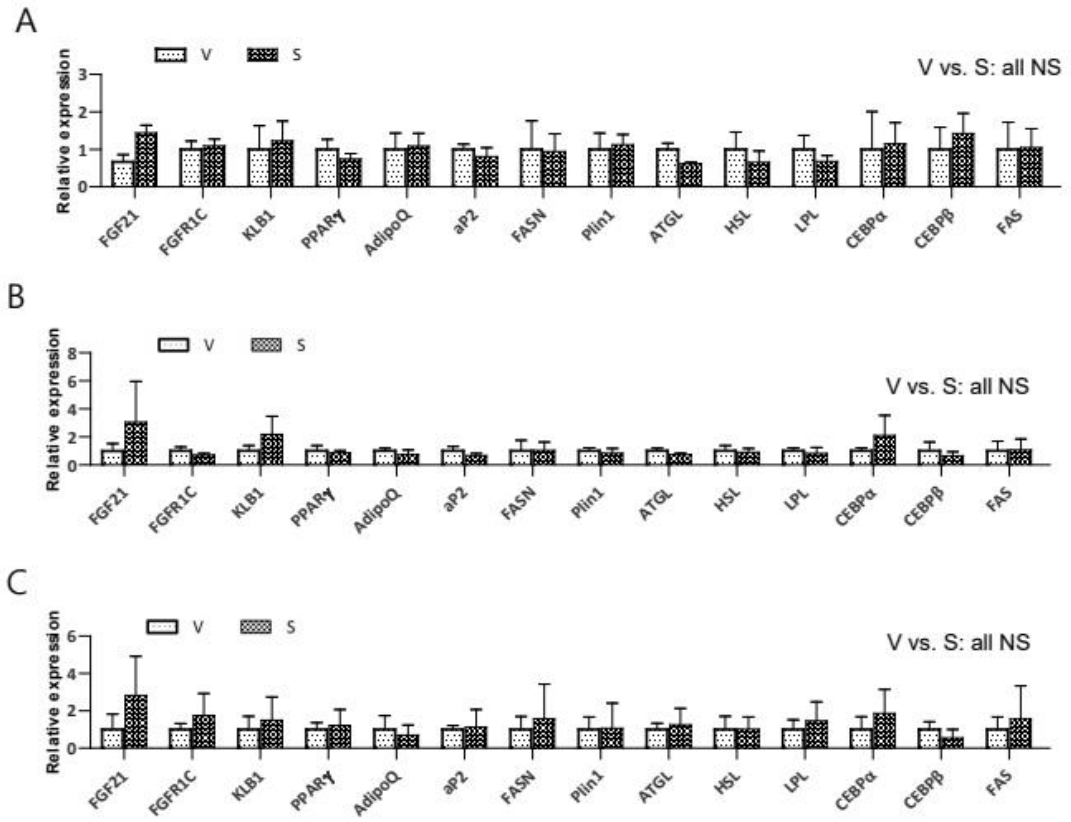
A: Western blot analysis, B: FGFR1, C: β-klotho, D: p-P38, E: p-ERK. V, VAT: visceral adipose tissue, S, SAT: subcutaneous adipose tissue. n=5/group. NS: nonsignificant.

Figure 5. Protein expression of the FGFR dimer and signaling components in visceral and subcutaneous fat tissue of the type 2 diabetes mellitus group of subjects who underwent coronary artery bypass graft surgery (Cohort 2)



A: Western blot analysis, B: FGFR1, C: β-klotho, D: p-P38, E: p-ERK. V, VAT: visceral adipose tissue, S, SAT: subcutaneous adipose tissue. n=5/group. a,b Significant differences ($P < 0.05$). NS: nonsignificant.

Figure 6. mRNA expression of FGFR dimer and signaling components in visceral and subcutaneous fat tissue



A: Non-diabetic control group of subjects who underwent general abdominal surgery (Cohort 3) B: Non-diabetic control group of subjects who underwent coronary artery bypass graft surgery (Cohort 2) C: Type 2 diabetes mellitus group of

subjects who underwent coronary artery bypass graft surgery (Cohort 2). V: visceral adipose tissue, S: subcutaneous adipose tissue. N=3/group. NS: nonsignificant.

Discussion

This study investigated the existence of the paradoxical elevation of plasma FGF21 in T2DM and the relationship between FGF21 concentrations and clinical parameters. Plasma FGF21 levels were elevated in patients with T2DM. Plasma FGF21 was strongly associated with increased TG, BMI, and insulin resistance.

FGF21 is suggested as a major metabolic regulator of glucose/lipid metabolism and obesity. FGF21 transgenic mice have improved metabolic profiles (cholesterol, TG, glucose control, and insulin sensitivity), and resistance to diet-induced weight gain and fat accumulation. In contrast, FGF21 deficiency led to increased body weight, development of fatty liver, impaired glucose tolerance, hypertriglyceridemia.

FGF21 can be detected in rodent and human blood. In animal studies, the systemic administration of FGF21 was found to result in a sustained decrease in blood glucose/TGs, improved insulin sensitivity, the amelioration of obesity/hepatosteatosis (Badman,

Pissios et al. 2007, Coskun, Bina et al. 2008, Xu, Lloyd et al. 2009), decreased LDL-C, and elevated of HDL-C (Kharitonenkov, Wroblewski et al. 2007), suggesting that FGF21 might have beneficial effect on metabolic diseases. However, ob/ob and DIO mice exhibit elevated FGF21 blood levels and are less sensitive to acute FGF21 administration (Berglund, Li et al. 2009, Fisher, Chui et al. 2010).

In human, FGF21 is paradoxically increased in diabetic, obese, and dyslipidemic individuals. Studies based on 232 Chinese subjects and 189 Korean subjects showed that FGF21 concentrations were significantly higher in obese subjects and correlated positively with adiposity, serum glucose, insulin, TG and components of the metabolic syndrome (Zhang, Yeung et al. 2008, Lee, Lim et al. 2014). In contrast, FGF21 levels in 17 anorexic women (mean BMI, 16 kg/m^2) were significantly lower than those in age-matched controls (Dostalova, Kavalkova et al. 2008). These findings lead to the suggestion of a compensatory mechanism and/or development of 'FGF21 resistance' in a metabolically compromised state.

In concordance with these previous studies, this study also showed

that plasma FGF21 levels are positively correlated with BMI, hypertriglyceridemia, and insulin resistance. Plasma FGF21 levels were significantly higher in T2DM than in NGT and prediabetes (cohort 1). Especially in the patients with severe coronary artery disease (cohort 2), plasma FGF21 levels were profoundly higher in T2DM than in non-diabetic control. The direct comparison of FGF21 levels between the 2 cohorts was not feasible. However, these results are in concordance with the suggestion that the FGF21 levels might reflect not only insulin resistant state but also coronary artery disease (Shen, Ma et al. 2013).

To further assessment of the FGF21 resistance in insulin resistant state, the regional fat amounts were measured in cohort 1. Ectopic fat has been suggested to play a key role in the development of insulin resistance and metabolic syndrome (Goodpaster, Krishnaswami et al. 2003, Kelley, McKolanis et al. 2003, Wang, Hsu et al. 2009). Therefore, the quantification of ectopic fat accumulation could have a substantial role in understanding FGF21 resistance.

Similar to a previous study (Goodpaster, Krishnaswami et al. 2003),

this study showed that all types of ectopic fat, and especially epicardial fat, were higher in the subjects with T2DM. Further, plasma FGF21 levels were positively correlated with all four ectopic fat accumulation indices in the subjects with T2DM, but not in NGT and prediabetes. Because the major target tissue of FGF21 is adipose tissue, this result suggests that the cause of paradoxical plasma FGF21 elevation in T2DM could be the altered metabolism in the ectopic fat.

Obtaining an accurate measurement of ectopic fat is not easy. To measure visceral fat amount, CT was used. Epicardial fat volume can be measured by CT; however, more effort is required because of its three-dimensional shape. Since manual segmentation of epicardial multi-plane CT image was performed, epicardial fat volume would yield most accurate results among the ectopic fat areas. These could be the reason why the epicardial fat volume showed a good correlation with FGF21 levels. In the assessment of intrahepatic fat and intramuscular fat, the gold standard is magnetic resonance spectrometry. However, because this technique is not commonly applicable, these types of fat could be measured by CT (Ricci, Longo et al. 1997, Goodpaster, Kelley et al. 2000).

Estimates of intrahepatic fat obtained by CT correlates to chemical measurement of liver TG obtained by liver biopsy (Shores, Link et al. 2011). The midthigh low-density muscle area was found to be well correlated with insulin resistance (Kim, Nam et al. 2003). Few studies have comprehensively measured ectopic fat accumulation in various sites in a single subject. However, in the present study, the evaluation of ectopic fat amount was conducted comprehensively at four different sites in each individual subject and the fat amounts were well correlated each other.

Regarding FGF21 resistance in genetic models, a reduced response to FGF21 treatment in a metabolically compromised state can be explained at the FGF21 receptor level, as FGFR1 and β-klotho transcripts are reduced in FGF21 responsive tissues in ob/ob and DIO mice, as compared to those in normal animals (Fisher, Chui et al. 2010).

In this study, human fat tissue grouped based on insulin resistance was examined to investigate the mechanism of FGF21 resistance. Components of the FGF21 receptor (FGFR1 and β-klotho) and proteins involved in post-receptor signaling (p-P38) were

significantly lower in VAT than in SAT in the subjects with T2DM. There were no significant differences of FGF21 receptors and post-receptor signaling between the VAT and SAT in non-diabetic control groups. Decreased FGF21 receptor expression and post-receptor signaling observed in visceral fat could result in FGF21 resistance in this disease. Moreover, it could be suggested that excessive ectopic fat accumulation, which is easily detected in patients with obesity, insulin resistance, and diabetes could contribute to the exaggeration of FGF21 resistance.

A previous study found that β-klotho levels were reduced in the visceral fat of high-fat diet (HFD) induced obese mice (Diaz-Delfin, Hondares et al. 2012). Further, a study on monkeys chronically fed an HFD demonstrated that HFD-resistant monkeys exhibited increased β-klotho levels in subcutaneous fat compared to those in HFD-sensitive monkeys (Nygaard, Moller et al. 2014). Moreover, elevated FGF21 in circulation and its action on adipose tissue lead to the accumulation of subcutaneous fat mass during diet-induced obesity (Li, Wu et al. 2018). The increase in β-klotho in subcutaneous fat and its decrease in visceral fat suggest that FGF21 preserves insulin sensitivity via its action on subcutaneous

fat (Li, Wu et al. 2018).

A recent paper suggested that FGF21 resistance could be indicated by using increased serum and/or tissue FGF21 levels, downregulation of receptor expression, impairment of downstream signaling cascade, and no response to exogenous FGF21 therapy (Tanajak 2017). The present study was conducted in cross-sectional human study, so therapeutic effect of FGF21 could not be shown. However, the other 3 components of FGF21 resistance in T2DM were well documented in the present study. Furthermore, consistent with other studies (Diaz-Delfin, Hondares et al. 2012, Nygaard, Moller et al. 2014, Li, Wu et al. 2018), decreased FGF21 receptor expression and post-receptor signaling observed in visceral fat could be one mechanism explaining the FGF21 resistance.

FGF21 also acts in an autocrine fashion in white adipose tissue to stimulate PPARγ activity (Dutchak, Katafuchi et al. 2012). Thus, the gene expression of FGF21 receptor dimer and PPARγ signaling components were investigated. The results were not reached to statistical significance between VAT and SAT. However, it showed

similar pattern of changes with respect to the protein expression of FGF21 receptors in T2DM patients.

The findings of this study present some clinical applications. First, the profound increase of FGF21 in severe coronary artery disease and positive correlation between FGF21 and epicardial fat amount imply the role of plasma FGF21 as a marker of cardiovascular disease. Preexisting studies also support this suggestion (Lin, Wu et al. 2010, Lee, Lim et al. 2014). Second, this study raises the plausibility that targeting ectopic fat FGF21 signaling may represent a therapeutic strategy to combat insulin resistance and T2DM.

This study has limitations. First, the gold standard of estimating intrahepatic fat and intramuscular fat is MR spectrometry. Thus, the analysis using intrahepatic fat and intramuscular fat could have statistically limited power. However, the assessment methods using CT were also validated. Second, this study was performed in relatively small numbers of Asian subjects. Although FGF21 level was not significantly different between males and females, the sex difference has the possibility to affect vias to the results. Large human studies in various ethnic backgrounds are required to

generalize this concept in human metabolic diseases. Third, the longitudinal change of FGF21 level was not investigated. It would be interesting to perform future study about the association between longitudinal changes of FGF21 and the progression of glucose metabolic derangement or weight change.

In conclusion, this study demonstrated that plasma FGF21 levels are higher in patients with T2DM. Plasma FGF21 is also positively correlated with ectopic fat accumulation in this disease and FGF21 receptor, FGFR1 and β-klotho, and post-receptor signaling, pP38, in visceral fat is attenuated. Thus, human FGF21 resistance in T2DM could be a result from down regulation of FGF receptor dimer and post-receptor signaling in ectopic fat accumulation. Our study supports to understand the mechanisms regulating FGF21 resistance and provides clinical insights to discovery of FGF21 therapeutics.

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요약 (국문 초록)

사람지방조직을 중심으로 한 fibroblast growth factor 21 resistance의 기전에 관한 연구

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이소성 지방 축적은 당뇨병, 대사 증후군, 심혈관 질환과 같은 대사 악화의 전형적인 특징이다. 섬유아세포 성장 인자 21 (Fibroblast growth factor 21, FGF21)은 간에서 주로 분비되어 지방 조직에 작용하는 새로운 대사 조절 물질로, 당대사 및 지질대사에 유리한 효과를 갖는다고 알려져 있다. 그러나, 혈장 FGF21농도는 제2형 당뇨병 및 비만에서 역설적으로 증가하여, 이 물질에 대한 저항성이 존재함을 시사한다.

이 연구는 인슐린 저항성이 있는 사람에서 FGF21 농도와 다양한 유형의 이소성 지방 축적 간의 상관관계를 조사하고자 하였다. 또한, 이 연구에서는 이소성 지방의 FGF21 수용체 발현 및 수용체 후 신호 전달을 조사하여 제2형 당뇨병 환자에서 나타나는 FGF21 저항성의 기전을 확

인하고자 하였다.

3 개의 독립적인 코호트가 조사되었다. 코호트 1은 건강검진센터에서 의뢰된 190명의 피험자로 구성되었다. 의무기록을 통해 신체계측, 임상 지표 및 병용 약물을 식별하고, 정상 혈당군, 당뇨병 전단계군 및 제2형 당뇨병군으로 나누어 분석하였다. 64- 슬라이스 다중 검출기 컴퓨터 단층 촬영을 이용하여 다양한 부위의 지방량 (피하, 내장, 심장 외막, 간 내 및 근육 내)을 측정하였다. 그리고 혈장 FGF21 농도를 측정하였다. 관상 동맥 우회 수술 (코호트 2) 및 일반적인 복부 수술 (코호트 3)을 시행한 피험자로부터 내장 및 피하 지방 조직을 얻었다. 제2형 당뇨병군 및 비당뇨병 대조군으로 나누어 각 지방 조직에서의 FGF21 수용체 발현 및 수용체 후 신호 전달을 분석하였다.

혈장 FGF21 농도는 체질량지수, 중성지방, HOMA-IR 및 Matsuda 지수와 유의한 관련성을 보였다. 혈장 FGF21 농도는 당뇨병 전단계 및 정상 혈당군에 비해 제2형 당뇨병군에서 유의하게 더 높았다. 제2형 당뇨병의 이소성 지방 축적 (내장, 심장 외막, 간 내 및 근육 내)은 정상 혈당군보다 유의하게 더 높았다. 제2형 당뇨병군에서 혈장 FGF21 농도는 이소성 지방 축적과 강한 양의 상관 관계를 보였다. FGF 수용체 이량체 (FGFR1와 β-klotho) 및 수용체-후 신호 전달 경로 관련 단백질 (p-P38)의 발현은 비당뇨병 대조군에서는 내장지방과 피하지방 조직

간의 차이가 없었지만, 제2형 당뇨병군에서는 피하 지방보다 내장 지방에서 유의한 감소를 보였다.

결론적으로, 혈장 FGF21 농도는 제2형 당뇨병 환자에서 더 높다. 또한, 혈장 FGF21은 제2형 당뇨병에서 이소성 지방 축적과 양의 상관관계를 가지고 있으며, 내장 지방에서 FGF21 신호전달과정은 약화된다. 따라서, 제2형 당뇨병에서 FGF21 저항성은 이소성 지방에서의 FGF21 수용체 이량체 및 수용체 후 신호전달의 하향조절에 의한 결과일 수 있다. 본 연구는 FGF21 저항성을 일으키는 기전을 이해하고 FGF21을 이용한 치료의 개발에 새로운 시각을 제시하였다.

주요어: 섬유아세포 성장인자 21, 이소성 지방, 제2형 당뇨병, FGF21 저항성

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